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Cloning of bovine intercellular adhesion molecules and characterization of expression of neutrophil adhesion molecules in periparturient cows and calves

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Cloning of bovine intercellular adhesion molecules and characterization of expression of neutrophil adhesion molecules in periparturient cows and calves

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ABSTRACT

The interactions of circulating leukocytes with the endothelium represent a key point in the effector functions of the immune system by regulating the specificity and strength of cell-cell interactions. The recruitment of neutrophils to sites of inflammation is initiated by chemoattractants. The initial contact of neutrophils on vascular endothelial cells is a rolling interaction mediated by members of the selectin family. These include E-selectin and P-selectin on the surface of activated endothelial cells, and L-selectin on neutrophils. The subsequent activation of leukocyte β_2 integrins which bind to the glycoprotein intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, is essential for firm adhesion of leukocytes to the blood vessel wall and subsequent extravasation into the surrounding tissue.

Some periparturient cows, as well as neonatal calves, have been known to be reduced in their capacity to respond to bacterial infection. In our first study, constituitive β_2 integrin (CD18) and L-selectin (CD62L) expression on neutrophils was determined from periparturient Holstein cows and neonatal calves by flow cytometry. Platelet-activating factor was used to activate neutrophils to measure up- and down-regulation of the adhesion molecules in vitro. Mean values for both constituitive and stimulated CD18 expression on neutrophils from cows and calves were highest at parturition, then declined during the first 15 hours postpartum on calf neutrophils, while CD62L declined dramatically by 9 to 15 hours after calving on cow and calf neutrophils. The constituitive CD18 and L-selectin levels on cow neutrophils recovered to prepartum values by one week postpartum. Calf neutrophil levels of CD18 expression recovered by one week of age and did not reach the original levels seen at birth whereas L-selectin expression exceeded birth levels by one week postpartum.

In the second and third experiments, bovine intercellular adhesion molecules, ICAM-1 and ICAM-3 were cloned, sequenced and analyzed. Bovine ICAM-3 sequence was derived from a bovine mammary gland cDNA library and a bovine lymph node cDNA library. The

cDNA sequence contains 1827 bp encoding for 544 aa. The deduced amino acid (aa) sequence from the coding region of bovine ICAM-3 shows 61% identity with human ICAM-3. The bovine ICAM-1 gene was obtained from a bovine endothelial cell cDNA library screened using a DNA fragment derived from bovine ICAM-3 gene. The 3398 bp bovine ICAM-1 sequence codes for 535 amino acids and shows 58% identity with human ICAM-1 and 47% identity with bovine ICAM-3 at the amino acid level. The predicted number and positions of cysteine residues in bovine ICAM-1 are all conserved among species including bovine ICAM-3. Both bovine ICAM-1 and -3 proteins consist of five immunoglobulin-like domains, thus aligning them with the immunoglobulin gene superfamily. Northern blot results show that the bovine ICAM-1 gene is expressed in stimulated leukocytes and bovine ICAM-3 predominantly in resting neutrophils.

GENERAL INTRODUCTION

Dissertation organization

The following dissertation is organized into a general introduction, three journal papers and a general conclusion. The general introduction is a review of the literature of adhesion molecules and immune status of periparturient cows and neonatal caves. The first paper is the research conducted to characterize the expression of adhesion molecules on neutrophils of periparturient cows and neonatal calves. The second and third papers are cloning, sequencing and analysis of intercellular adhesion molecules in cattle. The second paper had been accepted and the other two papers will be submitted for publication.

Literature review

Introduction

Periparturient and neonatal immunosuppression is suggested by the increased incidence and susceptibility of cows and calves to bacterial and viral infections during this period.[1-8] A key step in host defense in cattle is leukocyte recruitment to peripheral tissue following infection by invasive microorganisms. Leukocyte trafficking is an important part of tissue surveillance for infectious agents as well as a component of the mechanism for rapid leukocyte accumulation at sites of infection or tissue injury. The interaction of circulating leukocytes with endothelial cells is important for leukocyte recruitment.[9] The process of leukocyte recruitment into inflammatory sites begins with local dilation of postcapillary venules and margination of leukocytes to the endothelium of postcapillary venules.

Recruitment of neutrophils to sites of acute inflammation involves the coordinated function of multiple families of adhesion molecules, cytokines, and chemoattractants. A multistep model for this process includes a transient leukocyte adhesion between the leukocyte and endothelial cells of the vessel wall mediated by members of the selectin family, followed

by tight adhesion between the integrins and intercellular adhesion molecules on endothelial cells, resulting in tight leukocyte adhesion and arrest. Leukocytes then cross the endothelial lining of vessels and enter the peripheral tissues through interaction with platelet/endothelial cell adhesion molecule-1 (PECAM-1).[10] Selectivity in the process of leukocyte recruitment comes from the diversity of molecules capable of mediating each step.

The purpose of this review is to present the general knowledge of adhesion molecules, periparturient immunity in cows and calves, and their relationship for the maintenance of immunological defense during this critical time period.

Adhesion molecules

Selectins

The first step in the process of leukocyte recruitment involves the initial contact and loose interactions required for leukocyte rolling. The tethering of flowing leukocytes to the vessel wall and subsequent rolling are mediated by members of the selectin family.[11] The selectin family are heavily glycosylated, single chain integral membrane proteins that include P-, E-, and L-selectin.[12-14]

P-selectin (CD62P) is constituitively found in Weibel-Palade bodies of endothelial cells and in alpha granules of platelets.[15,16] It is mobilized to the cell surface within minutes after activation by thrombogenic and inflammatory mediators. Cell-surface expression of P-selectin by mobilization from intracellular storage granules is short-lived (minutes), and therefore is likely involved in the early phase of leukocyte rolling during inflammation. By contrast, E-selectin (CD62E) is neither synthesized constituitively nor stored within intracellular granules.[17] Expression of E-selectin by endothelial cells only occurs upon stimulation by cytokines with peak expression a few hours following inflammatory events, indicating "*de novo*" synthesis.[18] L-selectin (CD62L) is constituitively expressed on the surface of leukocytes and is rapidly shed by proteolytic cleavage upon activation.[19,20] L-selectin appears

to be crucial for recruitment of circulating neutrophils into inflamed tissue and lymphocyte homing into lymph nodes by slowing down leukocytes on contact with specific endothelial ligands prior to extravasation.[21,22] It has been reported that the cytoplasmic domain of L-selectin regulates leukocyte adhesion to endothelium independent of ligand recognition by controlling cytoskeletal interactions.[23]

During an inflammatory response, more P-selectin and E-selectin are expressed on endothelial cells stimulated by locally produced cytokines and interact with undefined counter ligands on leukocytes. It has been hypothesized that constituitively expressed L-selectin on leukocytes initiates margination through transient interactions with carbohydrate moieties of the selectins on endothelial cells.[24] However, the ligands for each selectin have not been elucidated clearly to date, although it is believed that ligands for selectins are diverse and complex macromolecules that share common types of anionic carbohydrates.[25] Neutrophils activated by cytokines or chemoattractants shed L-selectin, which is a prerequisite step for β_2-integrin-mediated adhesion. They also increase the functional activity of the β_2-integrin CD11b/CD18, leading to the association of cytoskeletal proteins with the cytoplasmic tails of integrin molecules, which are necessary for firm adhesion and spreading.[21,26] In a human genetic disease, termed leukocyte adhesion deficiency type 2 (LAD 2), a fucosylation failure reduces expression of all fucosylated lactosamines and is associated with markedly diminished selectin-mediated binding.[27] The patients of LAD 2 show a high blood neutrophil count, marked defects in neutrophil motility, recurrent pneumonia, and bacterial infection, which underlines the importance of the adhesive interactions mediated by selectins during the inflammatory response.[27]

Integrins

Integrins are the major family of cell surface receptors that mediate cell-cell adhesive interactions and attachment to the extracellular matrix. These integrin-mediated adhesive

interactions are intimately involved in the regulation of cellular functions, including embryonic development, maintenance of tissue integrity, as well as leukocyte recruitment and extravasation.[28-30] Structurally, they are heterodimers composed of non-convalently linked α and β transmembrane subunits selected from among 16 α and 8 β subunits that heterodimerize to produce more than 20 different receptors. In typical integrins, β subunits have a number of cysteines which form intramolecular disulfide bonds and the α subunits contain three to four repeats of a divalent cation binding motif.[31]

The β_2-integrins are mainly involved in leukocyte-endothelial cell contact. The β_2-integrin I (Inverted) domain, as well as the divalent cation binding site of the α chains are thought to be involved in ligand binding.[32,33] The β_2-integrin family consists of distinct α chains, CD11a, CD11b, and CD11c sharing a common β chain (CD18). Distribution of β_2-integrins on leukocyte surfaces varies with cell type and state of activation. LFA-1 (CD11a/CD18) is expressed on all leukocytes and is the only β_2-integrin expressed on T and B lymphocytes.[34] Mac-1 (CD11b/CD18) and p150,95 (CD11c/CD18) are predominantly found on granulocytes, with some expression on macrophages, and natural killer cells. They are stored in secondary and tertiary granules, and lead to a very remarkable increase in surface expression of these molecules following cellular activation due to translocation of intracellular granules to the cell surface.[34,35] The critical nature of normal β_2-integrin expression on leukocytes has been clearly demonstrated by failure of host defense in human patients and in animals with leukocyte adhesion deficiency (LAD) Type I.[36-39] Leukocytes in these patients fail to migrate from the peripheral blood into tissues in sufficient numbers to inhibit the growth of bacteria, and these individuals suffer recurrent, life-threatening infections.

Following activation of β_2-integrins, a divalent cation is required for ligand binding and cell adhesion.[40,41] The identity and concentration of cations present in the integrin microenvironment may affect its affinity for ligand and cellular regulation[41] by inducing conformational changes. For example, Ca^{2+} appeared to have an inhibitory effect on LFA-1

function[42] since binding to ICAM-1 occurs in the presence of Mg^{2+} or Mn^{2+}. However, Ca^{2+} binds with higher affinity to integrin cation binding sites, and may serve to maintain an inactive state during non-inflammatory conditions.[42]

Most activators of integrin binding also stimulate intracellular signal transduction pathways. The cytoplasmic domains of the β_2-integrins include phosphorylation sites that are accessible to these events and thus may mediate inside-out signaling and extracellular conformational changes.[43] Conformational changes of LFA-1 may be brought about either by binding by monoclonal antibody that activates LFA-1[44] or by intracellular elements with the carboxyl terminal cytoplasmic tail of the β_2-integrin.[45,46] It is possible that interactions of cytoskeletal proteins with the cytoplasmic domains of integrins[47] as triggered by leukocyte stimulation might cause conformational changes and increases in integrin affinity. In addition, β_2-integrins are involved in phagocytosis, aggregation, superoxide production, H_2O_2 production, and intracellular killing of *Staphylococcus aureus*.[48,49] Thilaganathan et al. reported that pregnancy in women is associated with increased expression of CD11a on neutrophils, suggesting neutrophil activation during pregnancy.[50] Even though the adhesion molecule expression and function on neutrophils in cattle during the periparturient period has not been characterized, alteration of cell adhesion molecules and migration ability might contribute to alterations seen in neutrophil functions, thus contributing to their increased susceptibility to disease.

Intercellular adhesion molecules

Members of the immunoglobulin (Ig) superfamily of proteins on endothelial cells bind to β_2-integrins expressed on leukocytes and play an important role in strengthening adhesive interactions between leukocytes and endothelial cells of postcapillary venules. Intercellular adhesion molecule (ICAM) -1 and ICAM-2 on endothelial cells are products of distinct but homologous genes containing five and two Ig domains, respectively.[51-53] Immunoglobulin

domains 1 and 2 of ICAM-1 are involved in binding LFA-1[54] while Ig domain 3 of ICAM-1 mediates binding to Mac-1.[55] In addition, ICAM-1 is a ligand for the major group of human rhinovirus serotypes[54,56] and *Plasmodium falciparum*-infected erythrocytes also use ICAM-1 as an endothelial cell receptor by binding a site which partly overlaps the LFA-1-binding domain.[57,58] ICAM-1 is expressed at low levels on resting endothelial cells, but its expression is increased by various cytokines (IL-1, IFN-γ, and TNF-α), which is important for high affinity binding of leukocytes to postcapillary venules as a prelude to subsequent egress into inflamed tissues.[59-61] Epithelial cells and stimulated leukocytes have also been shown to express ICAM-1.[59] ICAM-2 is constituitively expressed on leukocytes and at a high level on resting endothelial cells, but its expression is not augmented by activation.[62,63] It has been proposed that ICAM-2 plays an important role for recirculation of resting lymphocytes.[62] In contrast to ICAM-1 and ICAM-2, ICAM-3 expression is absent on endothelial cells and is restricted to cells from lymphoid and myeloid lineages.[64,65] Heavily glycosylated ICAM-3 possesses five Ig-like domains similar to ICAM-1 and belongs to the Ig gene superfamily. It has been suggested that ICAM-3 is the major ligand for LFA-1 during the initiation of immune responses as it is highly expressed on resting leukocytes.[64,66] However, it could be involved in lymphocyte-endothelium interaction as it might competitively inhibit LFA-1 binding to ICAM-1[64] although ICAM-3 has never been found on endothelial cells. The distinguishing point compared to ICAM-1 and ICAM-2 is that ICAM-3 has serine residues in the cytoplasmic tail, which may cause different cytoskeletal changes and signal transduction pathways.[67] On cellular stimulation, human ICAM-3 is rapidly and transiently phosphorylated on serine residues.[68] Because of this phosphorylation, ICAM-3 may give different intracellular signals when ligand is bound. Therefore, it could be postulated that a physiologic role for ICAM-3 in leukocyte interactions is to act as a first counter-receptor initiating the interaction with β_2-integrins. The affinity for ligand binding could be lower than with ICAM-1 but ICAM-3 would be the initial counter-receptor because of its much higher expression.[64] The initial

interaction of ICAM-3 during interactions between leukocytes could increase the degree of activation of the β_2-integrins on both cells, thus facilitating the β_2-integrin-mediated cell binding to ICAM-1, and establishment of a more stable cell-cell interaction. Later, ICAM-3 molecules would be displaced from the site of intercellular contact, thus becoming available to reinitiate additional interactions with other β_2-integrin molecules on newly recruited cells.[69]

The fine tuning and selectivity of the inflammatory response relies on the combinatorial variety of adhesion molecules on endothelial cells and leukocytes, which are in part differentially expressed on different classes of leukocytes and further modified both on endothelial cells and leukocytes activated by a multiplicity of chemoattractants.

Periparturient cows

Cellular immunity

Well-documented cellular dysfunctions in immune response assays (neutrophil chemokinesis, respiratory burst, phagocytosis, lymphocyte blastogenesis, and serum concentration of immunoglobulins) have shown a marked reduction in the ability of dairy cattle to respond to invasive microorganisms during the periparturient period. [2,3,5,70,71] Although factors contributing to the increased susceptibility to intramammary infections have not been fully elucidated, impaired neutrophil recruitment among periparturient cows has been implicated in the high susceptibility of these cows to infectious disease such as mastitis.[72]

Recruitment of neutrophils is one of the first steps in the inflammatory response. For neutrophils to fulfill an adequate host defense, they must adhere to the endothelial cells of blood vessels, migrate through the vessel wall to the inflammation sites, recognize and ingest foreign organisms, and finally kill ingested pathogens. The migration involves the combined action of multiple families of adhesion molecules and chemoattractants to an infection site. Neutrophil extravasation initially depends upon adhesive interactions between L-selectin (CD62L) on neutrophils and E-selectin (CD62E) and P-selectin (CD62P) on endothelial cells

with their respective counter ligands.[12,20,25,73-76] These bindings can trigger arresting of neutrophils by enhancing expression and adhesiveness of another adhesion molecule, Mac-1 (CD11b/CD18), which is a member of the β_2-integrin family of leukocyte adhesion molecules.[28,77,78] Mac-1 on neutrophils tightly binds to its ligand on activated endothelial cells, ICAM-1,[53,55,79-81] allowing neutrophils to stop circulating and migrate along the endothelial surface and into tissues. The importance of β_2-integrin molecules for neutrophil recruitment in cattle was exemplified by the genetic disease, bovine leukocyte adhesion deficiency (BLAD). The leukocytes from BLAD calves have no β_2-integrin expression and thus neutrophils are unable to migrate to sites of infection,[82-84] resulting in high susceptibility to infections.

Hormone and cytokine changes

Results from *in vivo* and *in vitro* studies have indicated that shared ligands (peptide hormones, peptide neurotransmitters and cytokines) and receptors are used as a common language within and between the immune and neuroendocrine systems.[85] The synthesis of immune mediators and their receptors by cells in the brain and endocrine organs as well as the expression of neuroendocrine hormones and their receptors in tissues and cells of the immune system provide strong evidence for the concept of a bidirectionally integrated neuroendocrine-immune response.[86] Consequently, the nervous, endocrine and immune systems are anatomically and functionally interconnected.

Hormonal changes during the periparturient period have been reported[87-89] and likely contribute to the immunosuppression. Increased serum cortisol concentration adversely affects bovine neutrophil function and has been implicated as an important factor in certain bovine infectious diseases.[90,91] It has recently been shown that glucocorticoids affect adhesion molecules on neutrophils by reducing β_2-integrin and CD62L expression. Cortisol or dexamethasone induce a neutrophilia without an increase in immature neutrophils,[92] suggesting

that glucocorticoids decrease the marginating pool of neutrophils, thus reducing the efficiency of egress of neutrophils from blood to tissues.

Glucocorticoids have significant immunosuppressive and anti-inflammatory properties, including inhibition of endotoxin-stimulated cytokine production and gene expression.[93] The mechanism by which glucocorticoids cause immunosuppression has been recently reported.[94,95] Glucocorticoids inhibit NF-κB activity by inducing transcription of the IκBα gene, thus blocking secretion of cytokines such as IL-1, IL-8, and TNF-α,[96,97] which are known to up-regulate the expression of β_2-integrins and down-regulate CD62L expression on leukocytes. Since plasma cortisol concentrations are known to increase during the immediate periparturient period,[88,98] and dexamethasone reduces CD62L and CD18 expression on bovine neutrophils,[92] glucocorticoids could cause an increase in susceptibility to disease through their effects on adhesion molecules. Furthermore, intercellular adhesion molecule-1 expression on endothelial cells and binding with neutrophils is reduced by dexamethasone *in vitro*,[99] which could contribute to a decline in host defense and an increased susceptibility to disease postpartum by impeding neutrophil egress.

Other hormones such as estrogen and progesterone also increase as pregnancy progresses. Estrogen had minimal effect on bovine neutrophil function after administration of high doses of estradiol to steers.[100] In contrast, estrogen injections stimulated phagocytic ability of the equine neutrophils[101] and experimental elevation of estradiol-17β in blood to levels similar to before calving enhanced the phagocytic competence of bovine neutrophils.[102] In addition, an increase in lymphocyte mitogenic response towards the end of gestation was observed in cattle[2] and incubation of porcine lymphocytes in low concentrations of estrogen, comparable to the levels in early pregnancy, resulted in a significant increase in lymphocyte blastogenesis response.[103] However, supraphysiologic concentrations of estradiol were observed to suppress human lymphocyte blastogenesis.[104] High concentration of estrogens are reached a few days before parturition in cows and estrone is a major form of estrogen

instead of estradiol-17β.[89] Depressed responsiveness of blood lymphocytes upon stimulation with mitogen has been reported around parturition and the immediate postpartum periods of cows.[2,105,106] It has also been shown in rats that progesterone can suppress spleen immune cell function both *in vivo* and *in vitro*.[107] Thus, elevated levels of progesterone during pregnancy could also be one of the factors responsible for the immunosuppression. Suppression of human neutrophil chemotaxis by prolactin has been reported in patients with prolactin-secreting tumors.[108] Estrogen has many physiologic effects on cells including the capacity to upregulate progesterone receptors.[109] A unique feature of the hormone status of the female animal at the end of gestation is that estrogen levels are increasing to very high levels while progesterone remains elevated. It is possible that the increase in serum estrogen is indirectly responsible for periparturient immunosuppression by increasing progesterone receptor numbers in tissues, thus making tissues more susceptible to the immunosuppressive effects of progesterone levels that do not change.

Increased plasma concentration of the endogenous opioids, beta-endorphin and met-enkephalin during the periparturient period in cows may also affect immune function.[110] Plasma concentration of these opioids peaks at parturition and cows experiencing dystocia have significantly elevated concentrations of beta-endorphin several hours postpartum compared to normal cows. Significantly increased beta-endorphin concentration in plasma may affect host defense mechanisms.

It has been postulated that differential expression of cytokines might occur and the cytokine production change might contribute to the increased incidence of disease of periparturient cows.[111-113] Change in cytokine levels in mammary secretions during the periparturient period was reported.[114] Levels of IL-2 activity were lower and interferon (IFN) was not detectable in mammary gland secretions (colostrum) during the last week of gestation and at parturition when compared to levels detected two weeks prepartum, whereas tumor necrosis factor (TNF) increased gradually during that period. Sordillo and Peel[112] showed a

strong positive correlation between TNF-α production and fatal endotoxemia due to mastitis during the periparturient period. Furthermore, the ability of mononuclear cells from blood and especially from the mammary gland to produce TNF-α was enhanced during the immediate postpartum period.[113] Therefore, as parturition approached, a higher capacity to produce TNF locally in colostrum could contribute to more severe disease during mastitis, which may be a significant factor for explaining severe clinical mastitis associated with this critical period. Deficiencies in local immune responses during the periparturient period may be explained by changes in endogenous cytokine levels present in mammary gland secretions, leading to diminished immune cell function and increased susceptibility.

Little information is available on cytokine profiles in the plasma and placenta of periparturient cows. The cytokine profile of plasma might be different from that of the placenta. For example, no significant changes of plasma IL-8 concentration were observed during pregnancy or at parturition, whereas IL-8 concentrations in amniotic fluid are increased significantly during pregnancy in women.[115] Interleukin-1 was also not detectable in plasma throughout pregnancy while it is measurable in amniotic fluid during late pregnancy and parturition.[116,117] Interleukin-6 steadily increases towards parturition in plasma and amniotic fluid.[116] TNF-α levels in human amniotic fluid at term pregnancy are significantly high.[118] Contrary to the increased levels of inflammatory cytokines (TNF-α, IL-1, and IL-6),[119] interferon-γ expression by immunohistochemical analysis was intense in early pregnancy and lower at parturition,[120] suggesting that a low-level of inflammation may be a normal occurrence in the placenta and this process may induce the production of cytokines which may play a role in the regulation of parturition.

It has been postulated that pregnancy results in suppression of cell-mediated immune function and enhancement of humoral immunity. As pregnancy progresses, Th1 cytokines (interferon-γ and IL-2) decrease and Th2 cytokines (IL-4, IL-5, IL-6 and IL-10) increase.[121,122] Estrogen and progesterone play roles as suppressers of cell-mediated immune

responses and enhancers of humoral responses in mice.[123] The change of immune status develops in parallel with increased plasma corticosteroid levels as well as increases in estrogen and progesterone. Corticosteroids are also known to suppress cell-mediated immune responses and enhance humoral responses by suppressing the production of Th1 cytokines such as IL-2 and interferon-γ production.[124] This is contrast with what dexamethasone does in cattle, in which dexamethasone wipes out interferon-γ.[125] This shift is thought to permit the fetal-placenta to avoid rejection by the mother's cell-mediated immune attack. The complex hormonal changes in pregnancy is probably responsible for key messengers between the immune and endocrine systems. Thus, the periparturient period provides a complicated phenomenon in the role of neuroendocrine hormonal mechanisms in regulating immune responses.

Immune status in neonatal calves

Neutrophil function in neonates

Immature or inadequate host defense mechanisms have been cited as one of the most important causes of increased susceptibility of neonates to infection.[7] While the high susceptibility of neonates is partially due to functional immaturity of the humoral immune system, one of the most important abnormalities in the cellular host defense system of the neonate is neutrophil dysfunction.[8] Polymorphonuclear cells, mainly neutrophils are the primary mediators of acute inflammatory responses and thus represent the most important acute defense against invading bacteria and fungi. Neutrophil functional deficiencies have been documented in both human infants[126-129] and newborn calves,[130,131] suggesting neutrophil defects may contribute to the increased susceptibility to infection in neonates. Neonatal calves, like human infants, are highly susceptible to infection. Diarrhea, septicemia, endotoxemia and bronchopneumonia are major causes of illness and death. Millions of calves die from

pneumonia alone every year in the U.S. and calf mortality is especially high in the first week of life.[132]

Storage pool

The most clearly established dysfunction of human neonatal phagocytic defense is the diminished neutrophil storage pool and diminished ability of neonatal neutrophils to migrate to sites of infection.[133] The number of circulating neutrophils increases rapidly in premature and term infants after birth, reaching a peak at 12 to 14 hours probably due to increased amounts of circulating colony-stimulating activity in the first days of life[134] and declining by 72 hours to values similar to those in adults.[135] Bovine neonates show a similar pattern in that the highest neutrophil counts are observed in calves after 12 hours of age followed by a decrease of cell counts over the next 48 hours.[136] More rapid depletion of neutrophils may also reflect diminished survival of human neonatal neutrophils, which has been observed *in vitro*.[137] The storage pool in neonates was approximately 10% of that in adults, and inflammatory stimulation or infection caused a greater release and depletion of neutrophil stores in neonatal rats.[138] All of these studies suggest that the ability of the neonate to increase neutrophil production in response to infection would be limited, which may be an important factor in neonatal susceptibility to infection.

Chemotaxis

Arrival of neutrophils from the circulation to the site of inflammation by chemotaxis is necessary for appropriate defense against infection. Chemotaxis is achieved through specific cell surface receptors by chemotactic agents such as formyl-methionyl-leucyl-phenylalanine (fMLP) derived from microbes, C5a, leukotriene B_4, and IL-8 from host. fMLP receptor concentration (cattle lack fMLP receptor) and binding kinetics in human neonates are equivalent to adult values but chemotaxis of neonatal neutrophils was less than that of adult neutrophils.[8] For example, the influx of neutrophils into the peritoneal cavity in response to fMLP, group B

streptococci or *E. coli* was remarkably reduced in neonatal rat compared with adults.[139] Human neonate neutrophil chemotaxis was also more severely impaired when low concentrations of activated serum[140] was used as chemotactic stimuli. However, bovine neonate neutrophils demonstrated enhanced chemotaxis, which indicated hypermotility of these cells *in vitro*[141] and *in vivo*,[142] implying that reduced mobilization of neutrophils at least does not seem to be a factor contributing to the susceptibility of newborn calves to bacterial infection.

F-Actin

Cytoskeletal activation is crucial for locomotory and phagocytic leukocyte functions and actin is one of the most abundant contractile proteins of the neutrophil motors. The polymerization of globular (G)-actin to filamentous (F)-actin represents a crucial step within the cytosketal activation of migration by neutrophils. Neutrophils from human infants have higher basal F-actin levels than those from adults, but F-actin generation upon stimulation is reduced in comparison with adults,[143,144] and even more fail to deform normally.[8] In contrast, basal F-actin values of bovine neonate and adult neutrophils were comparable.[145] In addition, F-actin polymerization and F-actin content of neonate neutrophils stimulated with zymosan-activated serum and other stimuli[146] was similar or even higher and more sustained than adult neutrophils.[145]

Phagocytosis and killing of bacteria

Neutrophils from healthy human and bovine neonates bind and ingest bacteria with efficiency similar to cells from adults although neonatal neutrophils ingest less efficiently under suboptimal conditions such as limiting concentrations of opsonins.[7,147] This may be relevant in that neonatal serum is frequently deficient in opsonins. Since efficient phagocytosis of microorganisms generally requires opsonization by both C3b and immunoglobulin, opsonic

activity measures the ability of serum to enhance the phagocytosis of an organism or particle. Low opsonic activity of neonatal serum has been related to impaired phagocytosis by both bovine and human neonatal neutrophils.[7,147]

Production of toxic oxygen metabolites such as superoxide anion (O_2^-) through the respiratory burst represents an important mechanism by which neutrophils destroy microorganisms. Dissimilarities in the respiratory burst leading to O_2^- between newborn calves and adult cows have been reported.[130,148] Significantly reduced production of O_2^- has been observed in resting neutrophils and neutrophils stimulated with phorbol 12- myristate 13-acetate and other agonists but not with opsonized zymosan from newborn calves.[130,131,149] The possibilities for reduced production of O_2^- in neutrophils of neonatal calves are abnormal PKC activation,[150] difference in cell size between neutrophils from newborn calves and adult cows,[151] or serum factors such as corticosteroids during the first few days of life or defective receptor signal transduction. In contrast, the generation of superoxide anion by human neonatal neutrophils is similar to that of adult cells.[152] Killing of ingested gram-negative and positive bacteria by neutrophils from human neonates has been normal in most studies. However, variable and usually mildly decreased bactericidal activity has been noted against certain bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* and deficits in killing by human neonatal neutrophils were found to be more apparent at high ratios of bacteria to neutrophils. Discrepancies were reported within the literature in killing ability of neonatal calf neutrophils, however, this may be due to use of various stimuli to activate cells.[130,148]

CD18 and L-selectin (CD62L) expression

Human neonatal neutrophils have shown distinct differences in the mechanisms of adhesion to endothelial cells compared with adult neutrophils. The amount of LFA-1 (CD11a/CD18) on the surface of human neonatal neutrophils was not different from adult cells and levels of LFA-1-dependent adhesion for neonatal neutrophils were the same as adult

neutrophils.[153] In contrast to LFA-1, constituitive expression as well as the chemoattractant-stimulated increase in surface expression of CD11b/CD18 on neonatal neutrophils are deficient compared with that of adult neutrophils,[153,154] which contributes to impaired CD11b/CD18-dependent adherence and migration of neonatal neutrophils.[129] Also, there is reduced mobilization of CD11b/CD18 from intracellular stores, and a lower cellular content of CD11b/CD18 than adult neutrophils.[153,155,156] A marked reduction in expression of CD62L was found on the surface of human neonatal neutrophils, which correlates with a reduced ability of human neonatal neutrophils to adhere to endothelial cells *in vitro*.[157] However, other studies of CD62L levels on human fetal neutrophils indicated CD62L levels appeared to be similar to those of adults,[158] which raises the possibility that stimulating factors in neonatal plasma may be responsible for the reduced L-selectin levels. Granulocyte-macrophage colony-stimulating factor, known to activate shedding of CD62L from adult neutrophils *in vitro*,[159] was elevated in cord blood of one of the studies[160] and it may be one of the factors contributing to decreased CD62L levels.

On the other hand, no significant difference in the constituitive and stimulated β_2-integrin expression on neutrophils was reported between bovine neonate and adult.[161] Moreover, neonatal bovine neutrophils have been reported to be as capable as adult neutrophils of endothelial transmigration *in vitro*. Surprisingly, in contrast to the similar effects of zymosan-activated serum and C5a chemotaxin on both neonate and adult neutrophils, neonatal bovine neutrophils responded to IL-8 with a greater rate of migration than adult neutrophils.[161]

Neonatal hormone levels

It has been established that cortisol has an immunosuppressive effect on lymphocyte and neutrophil function in cattle[90,91] and may have similar effects on leukocytes in neonatal calves.[162,163] Plasma concentrations of cortisol are highest at birth followed by a sharp decrease during the first 6 hours after calving and reach a plateau at 48 hours after birth,[164,165]

It has been shown *in vivo* and *in vitro* that cortisol in physiologic concentrations in calves can cause a significant decrease of T lymphocyte reactions induced by phorbol 12-myristate 13-acetate or concanavalin A because of the inhibitory effect of cortisol on IL-1 synthesis.[166] Plasma cortisol levels are also related negatively to gamma-globulin levels and T lymphocytes in neonatal calves.[167] Additionally, lymphocyte responses of colostrum-fed calves to mitogens were lower than colostrum-deprived calves,[162] which might suggest that factors absorbed from colostrum may also be important immunoregulators. Other hormones such as the opiate alkaloid concentration in plasma may also be immunosuppressive factors.[110]

Conclusion

The increased susceptibility of cows and calves to infectious disease during the periparturient period results in economic loss in the dairy industry. One of the most important defense mechanisms is the role of neutrophils in cows and calves during the periparturient period. Recruitment of neutrophils from blood to infected tissue involves multiple steps mediated by multiple adhesion molecules of leukocytes and endothelial cells. Hormones, as well as, cytokine changes occur in the cow and calf during the periparturient period and may alter leukocyte adhesion events and migration. Understanding the expression of neutrophil adhesion molecules during the periparturient period may provide clues about the relationship between adhesion molecules and neutrophil dysfunction during this critical time period. In addition, cloning and characterization of intercellular adhesion molecules in cattle will allow for further studies of inflammation that are currently not possible.

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EXPRESSION OF ADHESION MOLECULES ON NEUTROPHILS OF PERIPARTURIENT COWS AND NEONATAL CALVES

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Abstract

Leukocyte adhesion molecules are important in the initial stages of the inflammatory response. They mediate the adhesion of neutrophils to endothelial cells prior to emigrating into infection sites. It has been reported that some periparturient cows, as well as calves have a marked reduction in the capacity to respond to bacterial infections. An experiment was designed to determine the expression of the β_2-integrin (CD18) family of adhesion molecules and L-selectin (CD62L) on neutrophils from periparturient cows and neonatal calves. Constituitive CD18 and CD62L expression on neutrophils was determined by flow cytometry using specific monoclonal antibodies. Platelet-activating factor (PAF) was used to activate neutrophils *in vitro* to measure down-regulation of CD62L and up-regulation of CD18 on activated neutrophils. Mean values for both constituitive and PAF-stimulated CD18 expression on neutrophils from cows and calves were highest at parturition, then declined during the first 15 hours postpartum on calf neutrophils while CD62L declined dramatically by 9 to 15 hours after calving on cow and calf neutrophils. The constituitive CD18 and CD62L levels on cow neutrophils recovered to prepartum values by one week postpartum. Calf neutrophil levels of CD18 expression recovered by one week and did not reach the original birth levels, whereas CD62L expression exceeded birth levels one week postpartum. Cows exhibited a leukocytosis (neutrophilia) with a doubling of neutrophils at parturition which was inversely correlated with

CD62L expression on neutrophils. Low expression of CD62L on neutrophils from cows, and both CD62L and CD18 on calf neutrophils for several days postpartum may result in impaired inflammation, thus contributing to the increased susceptibility to disease in dairy cows and calves at this time.

Introduction

Neutrophils are the first line of cellular immune defense against pathogens and normal flora.[1] Phagocytosis and the killing of bacteria by neutrophils in conjunction with humoral factors are critical defense mechanisms of the mammary gland.[2,3] Recruitment of neutrophils to sites of acute inflammation involves the combined action of multiple families of adhesion molecules, cytokines and chemoattractants. Neutrophil extravasation initially depends upon adhesive interactions between L-selectin (CD62L) on neutrophils and E-selectin (CD62E) and P-selectin (CD62P) on endothelial cells with their respective counter ligands.[4-10] The binding of selectins to their ligands and chemoattractant release from endothelial cells can trigger arresting of neutrophils by enhancing expression and adhesiveness of another adhesion molecule, Mac-1 (CD11b/CD18), which is a member of the β_2-integrin family of leukocyte adhesion molecules.[11-13] Mac-1 allows neutrophils to bind tightly to activated endothelium via its ligand, intercellular adhesion molecule-1 (ICAM-1),[14-18] allowing neutrophils to migrate along the endothelial surface and into tissues along a concentration gradient of chemoattractant.[19-21] The importance of β_2-integrin molecules for neutrophil recruitment in cattle was demonstrated by the genetic disease, bovine leukocyte adhesion deficiency (BLAD). The leukocytes from BLAD calves have no β_2-integrin expression and thus neutrophils are unable to migrate to sites of infection.[22-24]

It has been well established that periparturient dairy cows are immunosuppressed.[25-28] Some periparturient and early lactation cows exhibit an increased incidence and severity of mastitis as a consequence of their inability to quickly mobilize neutrophils into the mammary

gland in response to infection.[29] Although factors contributing to the increased susceptibility to intramammary infections have not been fully elucidated, impaired neutrophil recruitment among periparturient cows has been implicated in the high susceptibility of these cows to mastitis.[30]

Immature or inadequate host defense mechanisms have been cited as one of the most important causes of increased susceptibility of neonates to infection.[31] The most clearly established defects of neonatal phagocytic defense are the diminished neutrophil storage pool and diminished ability of neonatal neutrophils to migrate to the site of infection.[32] In the newborn, various neutrophil functions are diminished, including opsonophagocytic activity, the filamentous actin content, as well as, actin polymerization after stimulation,[33] and chemotaxis.[34,35] Several studies examined the first step in the activation of neutrophils, their ability to adhere and to migrate through endothelial cell layers and showed that migration, which is dependent on the integrin adhesion molecule family to be abnormal.[36,37]

If neutrophils from periparturient cows and newborn calves have impaired abilities to migrate to sites of infection, it is likely that the initial adhesion molecule reactions might contribute to any impairment seen. The purpose of this study was to characterize the expression of two adhesion molecules, CD18 and CD62L, in periparturient Holstein cows and neonatal calves at birth and during the first month postpartum.

Materials and Methods

Animals and experimental design. Twelve periparturient Holstein cows were evaluated one month before their anticipated calving date through one month after parturition. Blood was taken by jugular venipuncture from each cow one to three times a week before parturition, three, nine, and fifteen hours after parturition, and then one to three times a week after parturition. Thirteen Holstein calves were obtained from the above twelve cows at birth and

blood samples were collected by jugular venipuncture right after birth. Calves were sampled on the same postpartum schedule as the cows.

Whole blood leukocyte counts and differentials. Total leukocyte counts were determined on blood collected in EDTA-containing vacutainer tubes by electronic counting (CellTrack; Angel Engineering Corp., CT). Slides for differential cell counting were prepared from whole blood smears that were stained with a combination of a Wright-Geimsa stain, and 200 cells were differentiated into band or segmented neutrophils, mononuclear cells, and eosinophils. The numbers of each cell population were calculated by multiplying their relative proportions in blood by the total leukocyte count.

CD18 and L-selectin immunostaining procedure. Resting and stimulated cells were stained for CD18 and L-selectin (CD62L) as previously described.[38] In brief, 100 µl of whole blood collected in acid citrate dextrose (ACD) vacutainer tubes was stimulated with platelet activating factor (PAF; 1-O-hexadecyl-2-acetyl-rac-glycero-3-phosphocholine; final concentration 1 mg/ml) (Sigma Chemical Co., MO) for 5 min at 39 C. Resting cells were similarly treated with saline instead of PAF. Ten µl of isotype control antibody (clone X-927, IgG1 isotype; DAKO Corp.,CA), human anti-CD18 (MHM23, LFA-1 β chain) monoclonal antibody (DAKO Corp., CA), and twenty µl of anti-CD62L antibody (clone DREG-56, IgG1 isotype; Pharmigen, CA) were added, incubated for 15 min at room temperature. All antibodies were directly conjugated with fluorescein isothiocyanate (FITC). Erythrocytes were lysed two times in 2 ml hypotonic lysing solution (10.56 mM Na_2HPO_4, 2.67 mM NaH_2PO_4, pH=7.34) for 1 min, restored in 1 ml hypertonic (3 x saline) restoring solution (10.56 mM Na_2HPO_4, 2.67 mM NaH_2PO_4, 0.43 M NaCl, pH=7.25), and then centrifuged for 5 min at 650 xg. The remaining erythrocytes were lysed by incubating samples for 10 min with 1 ml of 1:10 dilution of B&D lysing solution (Becton Dickinson, CA) and centrifuging at 650 x g for 5 min. The supernatant was discarded and samples were resuspended in 1 ml of isotonic salt

solution (0.126 M NaCl, 1.13 mM disodium EDTA, 5.365 mM KCl, 3.52 mM NaH_2PO_4, 23.34 mM Na_2HPO_4, 7.14 mM NaF, pH=7.52) (Coulter Diagnostics, FL).

Flow cytometric analysis. Surface-stained cells were analyzed in a flow cytometer (FACScan™, Becton Dickinson, CA) using Cell Quest™ acquisition and analysis software. Neutrophils were identified by forward and side scatter characteristics. Mean fluorescence intensities (MFI) were computed in the geometric linear mode and then converted into molecules of equivalent soluble fluorochrome (MESF) by using a calibration curve obtained by analyzing a preparation of beads conjugated with four different concentrations of FITC (Quantum™ 26, Flow Cytometry Standards Corp., NC).

Statistical Analysis. The mean and standard error of means (S.E.M) were taken from individual MESF values of CD18 and CD62L.

Results

Whole blood leukocyte counts and differentials. Figures 1 and 2. illustrate the total leukocyte, neutrophil, and mononuclear cell counts. As clearly demonstrated in Figure 1, there was a gradual increase of leukocytes as parturition approached, peaking at 9 hours postpartum and recovering two weeks after parturition in cows. Similarly, calves showed highest cell counts 7 hours postpartum, decreased by six days of age and then reached normal adult range two weeks after calving (Fig. 2). The increase was mainly caused by an increased number of circulating neutrophils before and after calving.

CD18 and L-selectin (CD62L) expression analysis. Basal CD18 expression on neutrophils of cows was increased before calving and peaked at calving compared to all other sampling times (Fig. 3). Expression of CD18 on blood neutrophils in all cows sampled declined after parturition. Figure 4 illustrates a gradual decline in CD62L expression during the two weeks before calving and then dramatically declined after calving. Basal CD62L expression gradually started to decrease 2 week prepartum, was lowest 7 hours after calving

and came back to normal range 3 weeks postpartum. These values actually exceeded those observed 3 weeks prepartum.

In calves, basal CD18 expression on neutrophils of all calves was highest at parturition when compared to the first month of life (Fig. 5). By 15 hours postpartum, there was a significant decrease in basal CD18 expression. The low level of CD18 expression persisted for two days postpartum and started to increase although CD18 expression never returned to birth levels. Calf neutrophil levels of CD62L were lowest 15 hours after calving, returned to the birth level within two days, and exceeded birth levels by one week of age (Fig. 6). Platelet-activating factor (PAF) stimulation of neutrophils produced parallel curves for CD18 and CD62L expression (Fig. 3 - Fig. 6). Interestingly, the ability of neutrophils to mobilize intracellular stores of CD18 from secondary granules to the cell surface was diminished at calving in both cows and calves compared to other times.

Discussion

Recruitment of neutrophils to an infection site is one of the first steps in the inflammatory response. Early and rapid neutrophil influx is important for defending against microorganisms. Dysfunctional neutrophil chemotaxis, iodination and chemiluminescence after calving have been reported.[28,39,40] Leukocyte emigration from blood into sites of inflammation involves the sequential interaction of adhesion molecules expressed by both leukocytes and endothelial cells. Transendothelial migration of neutrophils begins with leukocyte rolling, which is mainly dependent on selectins,[41] followed by activation of integrins, firm attachment to endothelium and migration across the endothelial surface.[42] We questioned in this study whether the adhesion molecules, CD18 and CD62L on neutrophils are expressed normally in periparturient cows. The incidence of infectious disease in periparturient cows and neonatal calves is quite high and is likely to be attributed to dysfunctional host defense mechanisms.

Changes in total and differential whole blood cell count have been reported on periparturient cows[28] and cows treated with synthetic corticosteroids.[38] Our data in this experiment was similar to previous studies, which demonstrated increased numbers of neutrophils in the circulation right before and after parturition. The possibilities for the increased output of neutrophils are from the bone marrow storage pool by granulocyte macrophage-colony stimulating factor (GM-CSF) or granulocyte-colony stimulating factor (G-CSF) and high concentrations of plasma cortisol. The decreased number of leukocytes 12 hours after calving can be explained by neutrophil migration into the uterus.

It has been reported that high concentrations of plasma cortisol during the periparturient period contribute to immunosuppression in dairy cows[43] by increasing susceptibility to infectious disease such as mastitis,[28,44] pneumonia and gastrointestinal disorders. Burton et al. showed that *in vivo* glucocorticoid administration to Holstein cows induced dramatic down-regulation of CD62L and CD18 expression on blood neutrophils.[38] Since glucocorticoids cause leukocytosis and neutrophilia, which is well correlated with total leukocyte cell count one day after parturition, the decreased expression of CD18 and CD62L molecules on neutrophils could result from elevated cortisol concentration in blood at parturition. Although we did not measure the cortisol concentration in blood along with expression of adhesion molecules on periparturition period, numerous studies have demonstrated elevated cortisol concentrations at parturition which sharply decline afterwards,[45] which is inversely correlated with our finding on CD18 and CD62L expression. The mechanisms by which glucocorticoids cause immunosuppression has been recently reported.[46,47] The synthetic glucocorticoid, dexamethasone, causes an increased rate of IκBα protein synthesis by inducing the transcription of the IκBα gene, an inhibitor of NF-κB. The inhibition of the NF-κB activity would block cytokine secretion since NF-κB, a transcription factor, is a critical regulator of inflammatory cytokine genes such as IL-1, IL-8, and TNF-α,[48,49] which are known to up-regulate the expression of β_2-integrins and down-regulate CD62L expression on leukocytes.

In addition, NF-κB activation is required to express endothelial adhesion molecules such as E-selectin,[50] P-selectin,[51] vascular cell adhesion molecule-1 (VCAM-1)[52] and ICAM-1[53] during inflammation. The NF-κB/IκBα system might play an important role in regulating cytokine-induced leukocyte recruitment, adhesion, and transmigration across the vessel wall.

Plasma glucocorticoids in calves have also been reported to be high at birth and to sharply decrease within 6 hours after delivery and then reach a plateau by 48 hours after birth.[54] This is well correlated with our findings on the expression of CD18 and CD62L on neutrophils of calves after birth. Another possible explanation for the decreased expression of CD62L on calves is granulocyte macrophage colony-stimulating factor (GM-CSF) since rGM-CSF down-regulates the expression of CD62L on neutrophils[55] and the concentration of GM-CSF is elevated in cord blood[56] as well as in blood of the first day neonate in humans.[57]

Platelet-activating factor was used as an in vitro stimulus because it is known to induce the up-regulation of human neutrophil CD11b and CD18 molecules[57] and stimulate transendothelial migration of these cells in vitro.[58] It has been shown that infusion of PAF into the teat cistern of dairy cattle causes rapid leukocyte migration into the mammary gland.[59] In this experiment, PAF up-regulated CD18 expression between 25-34% throughout the experiment (data not shown), but interestingly, up-regulation of CD18 expression on neutrophils by PAF was lowest at parturition. One explanation of the lack of up-regulation of CD18 expression by PAF at parturition is that CD18 expression at calving time has already been up-regulated *in vivo* to some extent by translocation of intracellular granules located within secondary and tertiary granules[60] to the cell surface because increased expression of CD18 is most likely associated with Mac-1 (CD11b) molecules on neutrophils.

In light of our previous findings about the suppressive effects of dexamethasone on CD18 expression, the paradoxical high expression of CD18 at parturition concurrent with peak endogenous glucocorticoid levels is not easy to explain. At the end of gestation, the concentrations of many steroid and peptide hormones undergo dramatic changes; some of these

hormones could have opposing effects on CD18 expression with the resultant net effect being increased expression of the β_2-integrins on neutrophils. For example, estradiol and opioids (met-enkephalin and β-endorphin) in plasma increase towards term in normal pregnancy, peak at parturition, and decline the day following parturition.[61,62] On the other hand, expression of CD62L is entirely consistent with the changes being mediated by increased endogenous glucocorticoids. It is not known in cattle what effects elevated progesterone and estrogen levels have on leukocyte adhesion molecule expression.

Cortisol levels at birth could be an important contributing factor for susceptibility to disease because neonates born with high cortisol concentrations have shown a high incidence of disease. Hoyer et al. reported that calves born to cows experiencing dystocia have higher plasma cortisol concentration than calves requiring no assistance. Cortisol concentrations of calves were higher than cows,[63] which might explain why CD18 expression of calves are lower at birth through one day of age compared to cows.[38] Plasma cortisol concentrations are elevated at birth in calves and decrease significantly during first 48 hours of life,[54] which might be connected with our result that CD18 expression on neutrophils of calves decreased after parturition and persisted for two days while that of cows did not decrease. Possibly, the plasma cortisol concentration in neonatal calves is higher than that of cows.

Although expression of adhesion molecules is essential for neutrophil adhesion and migration, simple expression of these molecules does not determine the functional capacity of cells to adhere and migrate *in vivo*. Since it is known that hyperadherence for neutrophils is dependent not only on the quantity of CD11b/CD18 translocated to the cell surface[64] but also on altered conformational changes of CD11b/CD18 for its ligand under continuously increasing chemotactic stimulation,[65] further research is needed to determine the functional capacity of neutrophils of periparturient cows and calves to adhere and migrate during acute inflammation utilizing bovine endothelial cell cultures and chemotactic factors.

In summary, the decreased expression of CD62L on cow neutrophils and the CD18 and CD62L expression on calf neutrophils one day after parturition may result from elevated plasma cortisol concentrations, which could cause diminished egress of neutrophils from blood into tissue of cows and calves during the first day postpartum. This may be one of the factors contributing to the high incidence of infectious disease in these animals. The high expression of CD18 on neutrophils at parturition can be explained by complicated relationships among the endocrine and immune systems and their net effects on adhesion molecules. Dramatically decreased expression of CD62L on neutrophils at parturition may cause the diminished egress of neutrophils from blood into tissues of cows and calves.

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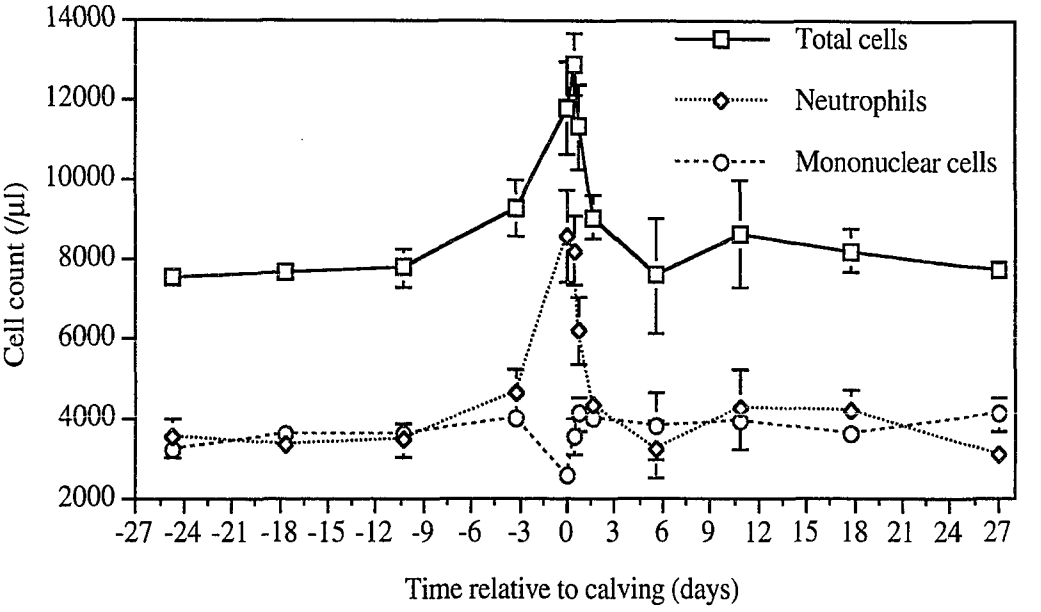

Fig. 1. Leukocyte count of periparturient cows. The vertical lines are SEM (n=4).

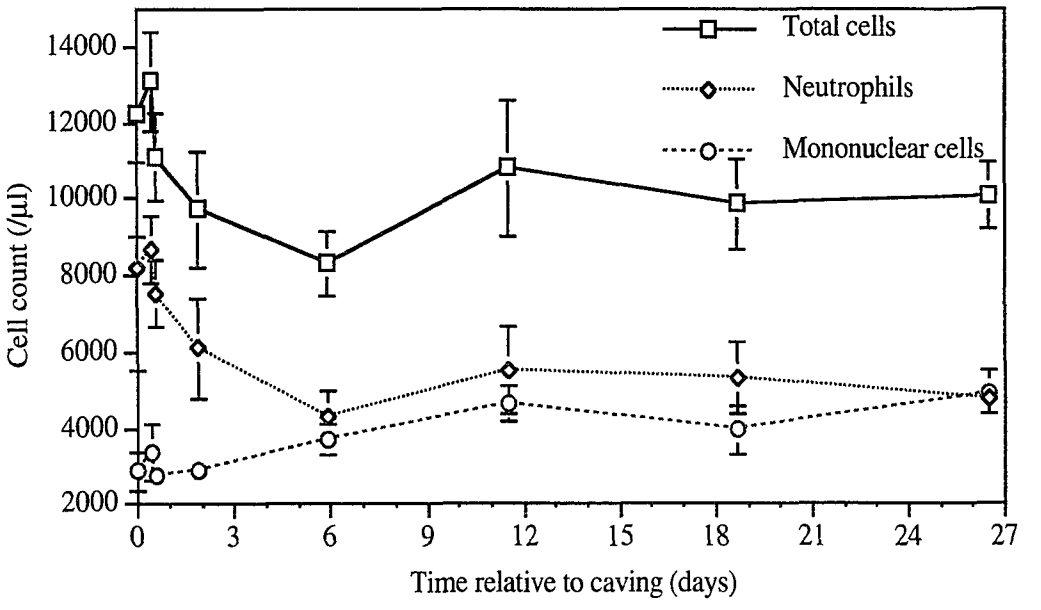

Fig. 2. Leukocyte count of neonatal calves. The vertical lines are SEM (n=4).

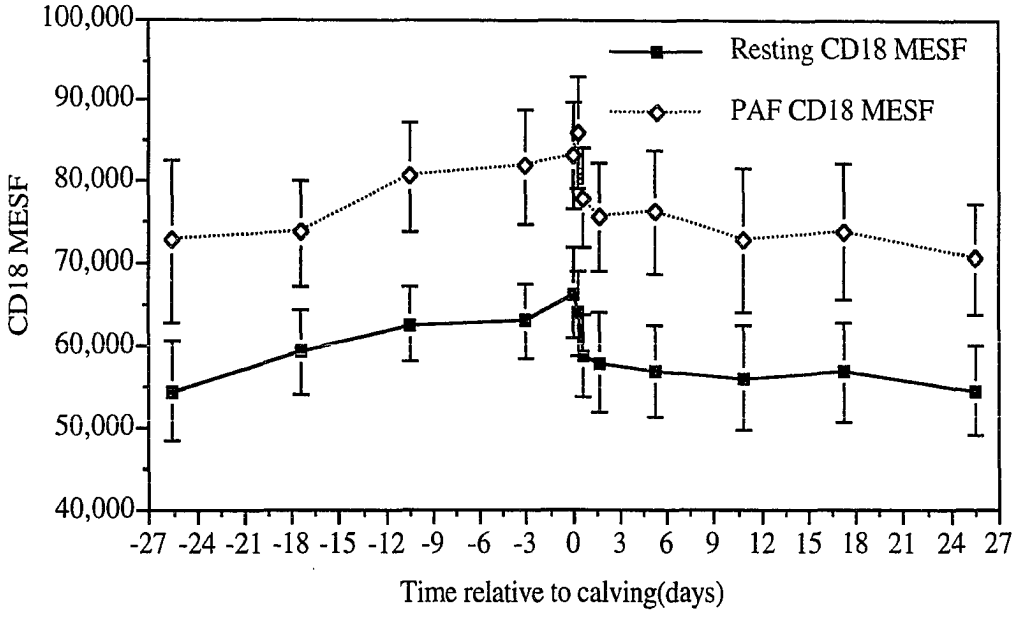

Fig. 3. Constituitive and PAF-stimulated CD18 expression on neutrophils of periparturient cows. The vertical lines are SEM (n=8).

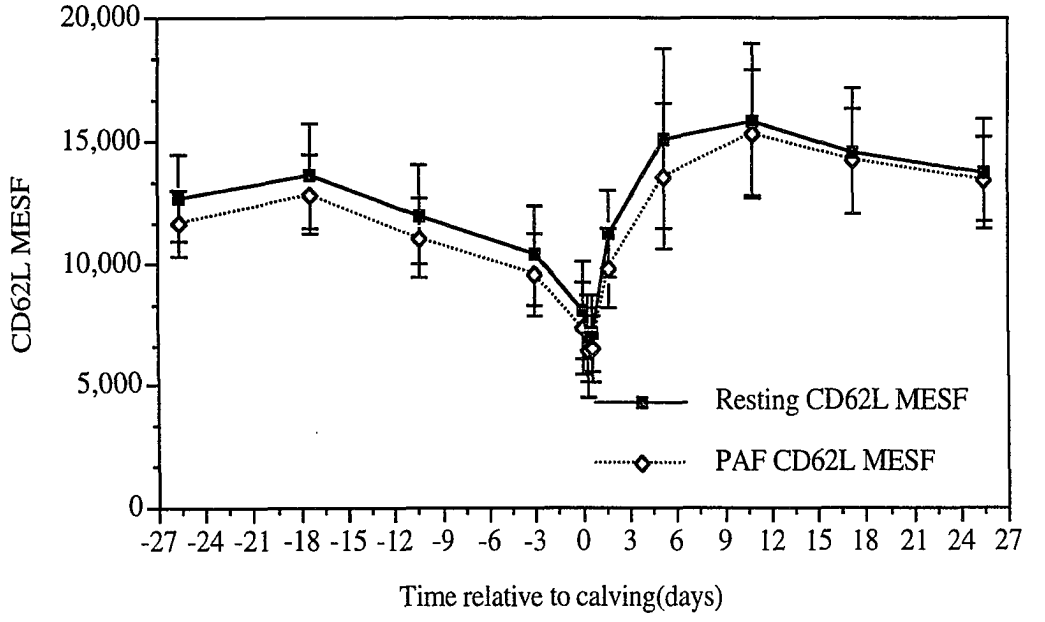

Fig. 4. Constituitive and PAF-stimulated CD62L expression on neutrophils of periparturient cows. The vertical lines are SEM (n=8). Mean fluorescence intensities were converted into molecules of equivalent soluble fluorochrome (MESF) by using a calibration curve obtained from beads conjugated with four different concentration of FITC.

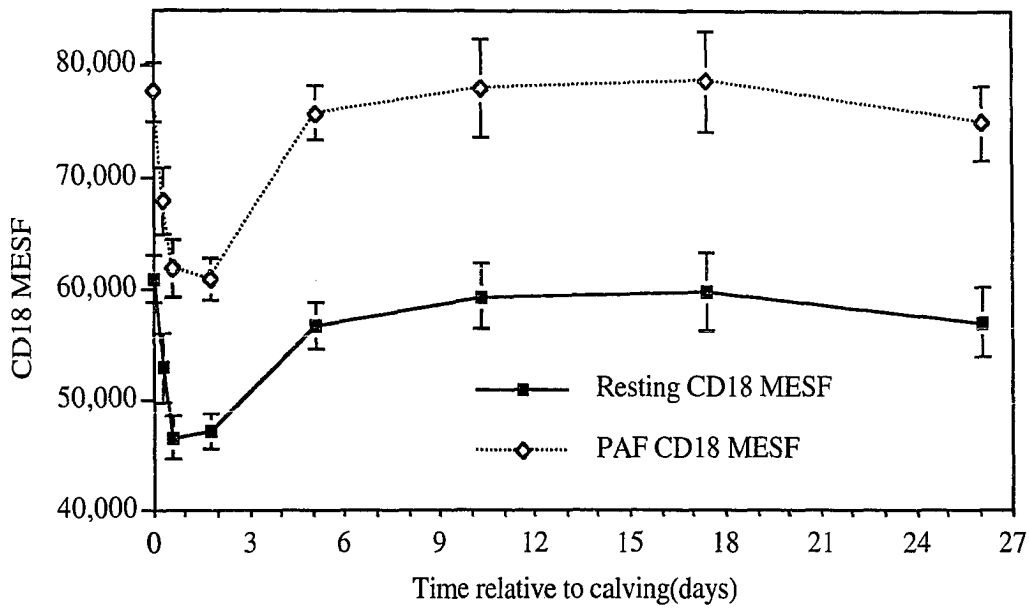

Fig. 5. Constituitive and PAF-stimulated CD18 expression on neutrophils of neonatal calves. The vertical llines are SEM (n=10).

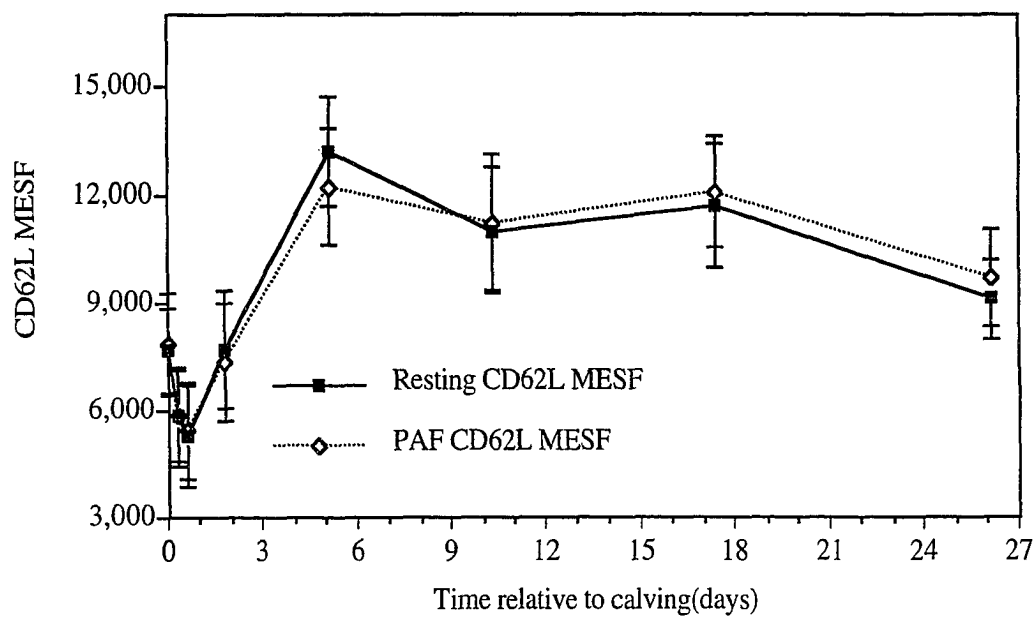

Fig. 6. Constituitive and PAF-stimulated CD62L expression on neutrophils of neonatal calves. The vertical lines are SEM (n=10). Mean fluorescence intensities were converted into molecules of equivalent soluble fluorochrome (MESF) by using a calibration curve obtained from beads conjugated with four different concentration of FITC.

CLONING AND SEQUENCING OF A CDNA ENCODING BOVINE INTERCELLULAR ADHESION MOLECULE (ICAM)-3

A paper to be published in *Gene*

Eun-kyung Lee, Marcus E. Kehrli Jr., Allan B. Dietz, Brad T. Bosworth and Timothy A. Reinhardt

Abstract

Intercellular adhesion molecule-3 is a ligand for the lymphocyte function-associated antigen-1 (LFA-1) on the surface of leukocytes. We cloned and sequenced bovine ICAM-3 and compared its sequence with human ICAM-3 and ICAM-1. A pair of consensus primers was prepared based on conserved sequences of ICAM-1 across several species. This primer pair amplified a 500 bp fragment that was used to screen a bovine mammary gland cDNA library. One positive clone contained a 1.5 kb insert and lacked about 150 bp at the 5' end of the coding region based on comparison with human sequences. The 5' end of the gene was amplified using bovine specific primers and plasmid specific primers from a bovine lymph node cDNA library. The bovine ICAM-3 shows 71% homology with human ICAM-3 and 64% homology with human ICAM-1 at the nucleic acid level. The overall homology of the deduced amino acid sequence with human ICAM-3 and ICAM-1 was 61% and 58% respectively. Bovine ICAM-3 has five immunoglobulin (Ig)-like domains, which indicates that it belongs to the Ig gene superfamily. The predicted number and positions of cysteine residues were all conserved between bovine and human ICAM-3 amino acid sequences. The fifteen potential N-glycosylation sites in the bovine ICAM-3 suggest that the protein is heavily glycosylated like human ICAM-3.

Introduction

The interaction of lymphocyte function-associated antigen-1 (LFA-1), on the surface of leukocytes, with its ligands is one of several regulated pathways involved in leukocyte cell/cell interactions.[1] Three ligands, ICAM-1, ICAM-2, and ICAM-3 for the LFA-1 integrin have been identified and sequenced.[2-5] Cytokine-activated endothelial cells express ICAM-1 as an inducible cell surface protein which is important for high affinity binding of leukocytes to postcapillary venules as a prelude to subsequent egress into inflamed tissues.[6] Epithelial cells and stimulated leukocytes have also been shown to express ICAM-1.[6] In contrast to ICAM-1, ICAM-2 is constituitively expressed on leukocytes and endothelial cells.[7,8] It has been proposed that ICAM-2 plays an important role for recirculation of resting lymphocytes.[7] Human ICAM-3 expression is absent on endothelial cells and is restricted to cells from lymphoid and myeloid lineages.[9,10] It has been suggested that ICAM-3 is the major ligand for LFA-1 during the initiation of immune responses as it is highly expressed on resting leukocytes.[9,11] ICAM-1 and ICAM-3 both possess five Ig-like domains, whereas ICAM-2 has two Ig-like domains, and all are members of the Ig gene superfamily. *ICAM-3* has only been reported for human while *ICAM-1* has been sequenced for many species such as human, dog,[12] mouse,[13,14] and rat.[15] We report here the cloning and sequencing of cDNA for putative bovine *ICAM-3*.

Materials and Methods

RNA preparation and cDNA synthesis. Total RNA from bovine leukocytes was isolated by TRIzol (Gibco BRL, Gaithersburg, MD) and chloroform, precipitated with isopropanol, and washed with 75% ethanol. Three μg of total RNA mixed with 0.5 μg of oligo-(dT) primer was heated at 70 C for 10 minutes . The annealed RNA was incubated for 1 hour at 45 C in a volume of 10 μl containing 10 mM dNTP, 200 U SuperScript II reverse

transcriptase and then a 5 µl of aliquot of the reverse transcriptase reaction was amplified (described below).

Primer design. Using primers based upon regions of homology among human, murine, chimpanzee and canine ICAM-1 cDNA, PCR was used to amplify a cDNA fragment from reverse transcribed bovine leukocyte RNA. Several sets of primers from domains 2 and 4 of ICAM-1 were prepared based on conserved sequences among known canine, mouse, rat, and human ICAM-1. One pair of primers, 5'-GTGGTGCTGCTCCGTGGG-3' and 5'-ACCTCGGTCCCTTCTGAGACCTC-3' amplified a 500 bp fragment from cDNA and had high homology with human ICAM-3 and -1 sequences.

Screening libraries and modified heminested PCR. This amplified DNA fragment was then used as a hybridization probe to screen a bovine mammary gland cDNA library. Screening of the cDNA library resulted in several positive clones, one of which contained a 1.5 kb insert but which lacked about 150 bp at the 5' end of the coding region based on comparison with the human sequence. The missing 5' end of the gene was amplified from a bovine lymph node cDNA library using a heminested series of lower specific primers for the 3' end and a library vector specific primer for the 5' end (Table 1).

Table I. Hemi-nested primer set used to obtain 5' end of bovine ICAM-3

No.[a]	Primers (5' to 3')	nt position at DNA
vecI	TAATACGACTCACTATAGGG	2999-3016 (or 3)
bovI	TGGTCCTCTCTTCTCGGCTGC	639-659
bovII	GAGCAGCACCACGGAGAGG	552-570
bovIII	GCCAGAGCAGAGAATTTCCAT	479-499
bovIV	CAATCTAAGGTGCAGTTTACCAC	253-275

[a] Library vector, pcDNA2 (Invitrogen) and bov ICAM-3 specific primers used sequentially in a 3' to 5' ordered sequence of reactions.

Results

cDNA cloning and sequencing. The composite cDNA sequence shows 71% identity with human *ICAM-3* and 64% identity with human *ICAM-1* at the nucleic acid level, suggesting the sequence to be bovine *ICAM-3*. The cDNA sequence of bovine *ICAM-3* contains 1827 bp with an open reading frame of 1635 nucleotides that encode for 544 aa (amino acids), starting with a methionine codon at nucleotide 101 and ending with a termination codon at nucleotide 1734 (Fig. 1).

Deduced amino acid sequence comparison. The deduced amino acid sequences from the coding region of bovine ICAM-3 show 61% and 58% identity with human ICAM-3 and -1, respectively (Fig. 2). Bovine ICAM-3 aa sequence contains a putative signal peptide, a hydrophobic transmembrane region, and a cytoplasmic tail. It also contains twelve cysteine residues at the same positions as those of human ICAM-3 and fifteen N-glycosylation sites, predicting heavy glycosylation like human ICAM-3. Similar to human ICAM-3 and -1, bovine ICAM-3 consists of five immunoglobulin-like domains, which aligns it with the Ig gene superfamily.

The conservation of amino acid sequences is high throughout all five domains of bovine ICAM-3 compared to human ICAM-3. Domain 4 is the most highly conserved domain between human and bovine ICAM-3 amino acid sequences (72% identity), whereas domain 2 of ICAM-1 sequences among species is the most highly conserved domain. The homology of the transmembrane region and cytoplasmic tail between species for ICAM-3 was 36% and 38%, respectively, which is lower than that of extracellular domains. The putattive bovine ICAM-3 like human ICAM-3, has three serine residues.

Discussion

ICAM-3 is one of three ligands for the leukocyte β_2-integrins. It has been speculated that ICAM-3 may play an important role in initiating immune responses because it represents the major β_2-integrin ligand on resting lymphocytes.[9] Bovine and human ICAM-3 have serine residues in the cytoplasmic tail, whereas neither human, canine, rat, nor mouse ICAM-1 or -2 contain any serine residues in their cytoplasmic tails. The low homology of the cytoplasmic region of ICAM-3 compared to ICAM-1 or -2 may cause different localizations and activate different signal transduction pathways on the cell surface.[16] On cellular stimulation, serine residues on human ICAM-3 become rapidly and transiently phosphorylated.[17] Because of this phosphorylation, ICAM-3 may give different intracellular signals when ligand is bound, which may result in different roles in cell-cell adhesion reactions. Although all three ICAMs are ligands for LFA-1, each may have distinct binding sites for LFA-1 or result in different signal transduction, which may lead to different immune response pathways.

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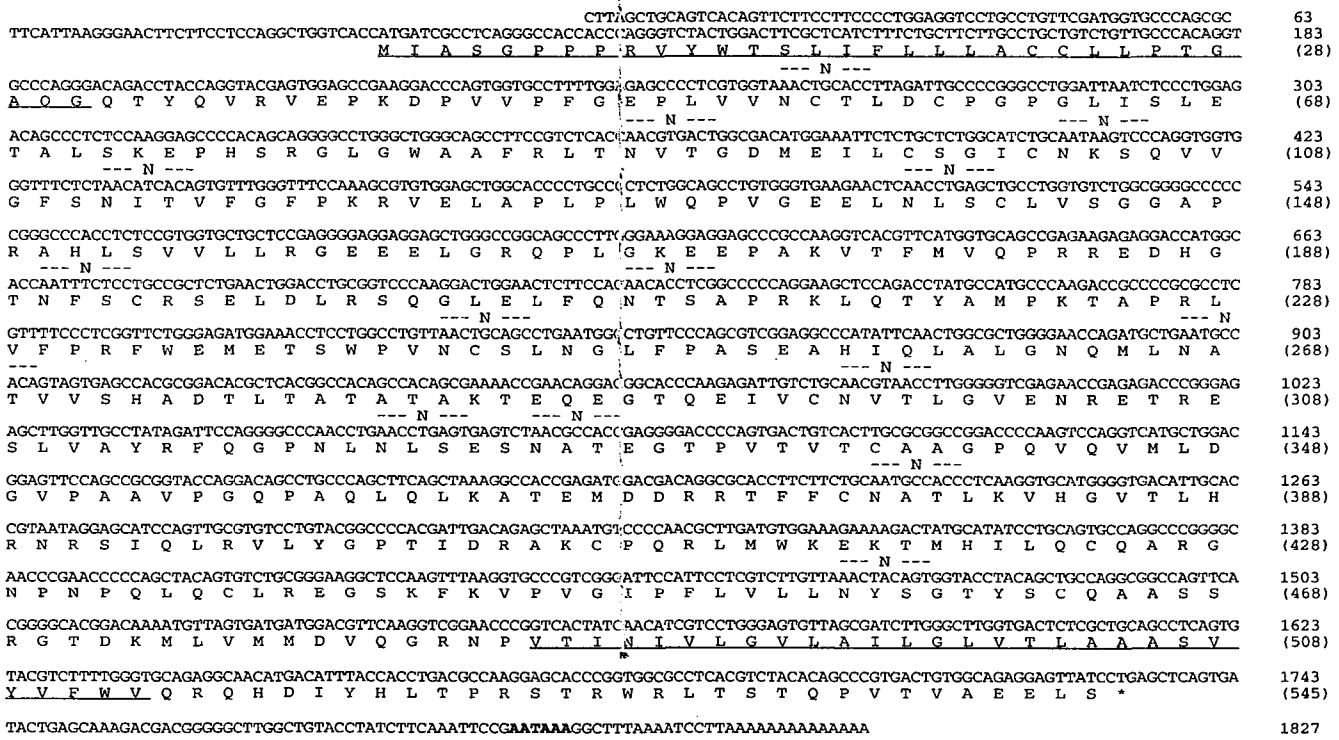

Fig. 1. Nucleotide seqence and predicted translated amino acid sequence for bovine ICAM-3 cDNA. The hydrophobic putative signal peptide and transmembrane region are underlined. Potential N-glycosylation sites are indicated by --- N ---. The polyadenylation signal sequence (AATAAA) in the 3'-untranslated region is in bold. The nt sequence data have been deposited with the GenEMBL Database (accession No. L41844).

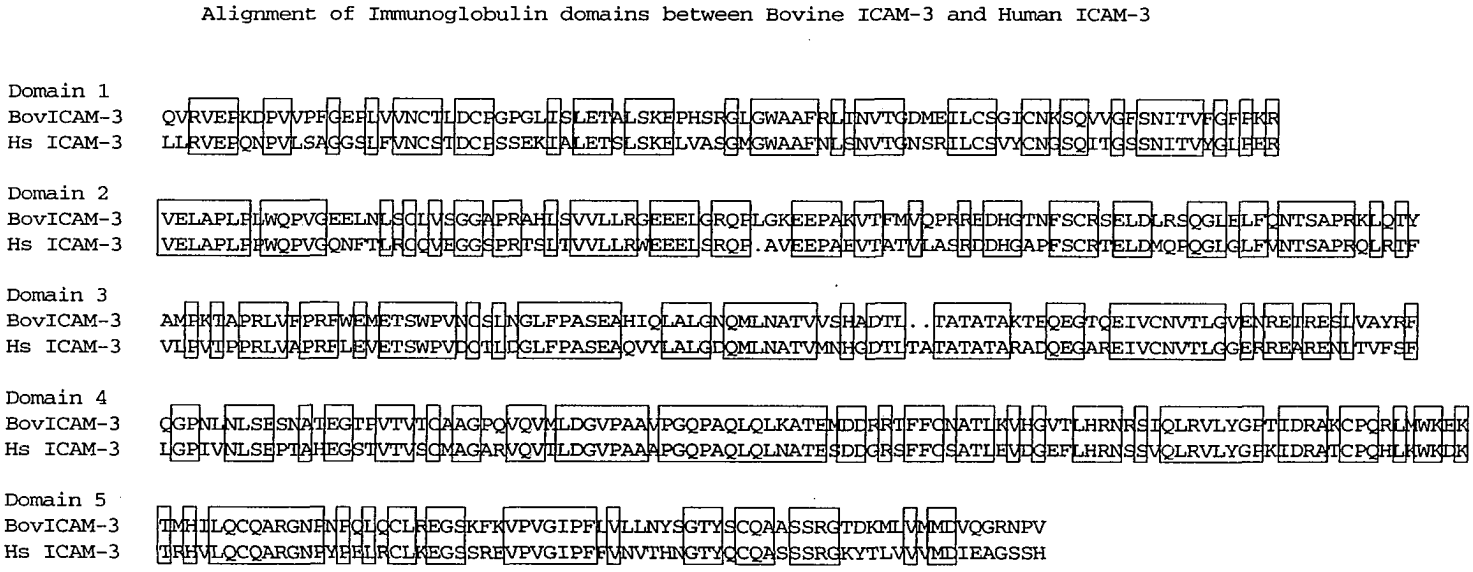

Fig. 2. Amino acid comparison between bovine ICAM-3 and human ICAM-3. Alignments were achieved using the program BESTFIT from the GCG (Genetics Computer Group) programs. Boxed amino acids indicate identity between bovine and human sequences. Period (.) was inserted to achieve maximum alignment homology. The letters are single letter designation for amino acids.

CLONING, SEQUENCING AND ANALYSIS OF CDNA ENCODING BOVINE INTERCELLULAR ADHESION MOLECULE-1 (ICAM-1)

A paper to be submitted to *Gene*

Eun-kyung Lee, Sang-Gu Kang, and Marcus E. Kehrli Jr.

Abstract

Intercellular adhesion molecule-1 (ICAM-1) is an inducible glycoprotein that interacts with the leukocyte β_2-integrins, LFA-1 and Mac-1. We have isolated and analyzed a cDNA clone coding for the putative bovine ICAM-1 gene and compared it with known comparative sequences from other species as well as bovine ICAM-3. The 3398-bp bovine ICAM-1 cDNA sequence codes for 535 amino acids and shows 57% homology with human ICAM-1 and 47% homology with bovine ICAM-3 at the amino acid levels. The predicted number and positions of cysteine residues in bovine ICAM-1 are all conserved among species including bovine ICAM-3. It has two arginine-glycine-aspartate (RGD) sites in the extracellular region and a serine residue in the cytoplasmic tail. Northern blot results show that the bovine ICAM-1 gene is expressed in stimulated leukocytes whereas bovine ICAM-3 is expressed predominantly in resting neutrophils.

Introduction

Intercellular adhesion molecule (ICAM)-1 is a cell surface glycoprotein with five immunoglobulin (Ig)-like domains. It plays an important role in interactions between leukocytes and in transendothelial migration of leukocytes through its expression on vascular endothelium by interacting with the β_2-integrins, LFA-1 (CD11a/CD18) and Mac-1

(CD11b/CD18) on leukocytes.[1-3] ICAM-1 is constituitively expressed on hematopoietic cells, vascular endothelium, fibroblasts and some epithelial cells, and its expression is upregulated in inflammatory responses[4] while ICAM-3 expression is restricted to cells from lymphoid and myeloid lineages.[5,6] It has been reported that Ig domains 1 and 2 of ICAM-1 mediate binding to LFA-1 and Ig domain 3 of ICAM-1 is involved in binding to Mac-1.[7,8]

Leukocyte emigration from blood to sites of inflammation involves the sequential interaction by adhesion molecules expressed by both leukocytes and endothelial cells. Transendothelial migration of leukocytes begins with leukocyte rolling, which is mainly dependent on selectins,[9] followed by activation of integrins, firm attachement to endothelium, migration across the endothelial surface to intercellular junctions and the subsequent migration between endothelial cells.[10] Integrin binding to ICAM-1 is particularly important for firm attachment and migration across endothelium. It has been postulated that ICAM-3 is potentially the most important ligand for LFA-1 in the initiation of the immune response because the expression of ICAM-1 on resting leukocytes is low and ICAM-3 is high.[5,11]

The cDNA of ICAM-1 for many species such as human,[12,13] dog,[14] mouse,[15,16] and rat[17] has been reported. The purpose of this paper is to report the cloning and sequencing of a cDNA encoding bovine ICAM-1 and comparative sequence analysis with other species. Gene expression of bovine ICAM-1 and ICAM-3 is also studied in resting and stimulated leukocytes.

Materials and Methods

Preparation of cDNA probe for screening the library. One pair of primers, 5'-CCTCTCCGTGGTGCTGCTC and 5'-GCAGTTAACAGGCCAGGAG, from Ig domains 2 and 3 of the cDNA encoding bovine ICAM-3 was designed[18] to amplify 280 bp of DNA, which has high homology with cDNA of human ICAM-1 based on sequence analysis. The PCR product was labeled with ^{32}P-dCTP using nick translation (Pharmacia, Piscataway, NJ) and used as a probe to screen a bovine endothelial cell cDNA library.

cDNA library screening and clone isolation. A Uni-ZAP™ XR cDNA library (Stratagene, La Jolla, CA) of bovine endothelial cells treated with recombinant human tumor necrosis factor-α was used for screening. Plaque lifts and phagemid *in vivo* excision were carried out according to manufacturer's protocols. Plaque hybridization was performed overnight at 42°C in a solution containing 50% formamide, 6 x SSC, 3.3 x Denhardt's solution, 25 mM Na_2HPO_4/NaH_2PO_4 solution, 0.1 mg/ml sonicated salmon sperm DNA, and 0.4% SDS. Filters were washed twice in 2x SSC/0.1% SDS for 5 min at room temperature (RT), once in 0.2x SSC/0.1% SDS for 10 min at RT, twice in 0.1x SSC/ 0.1% SDS for 5 min at RT, and final washing in 0.1x SSC/0.1% SDS for 5 min at 60°C, then exposed at -70°C for 18 h to X-ray film for autoradiography. Three rounds of plaque hybridization were done to enrich for positive clones After the third screening, the λ clones were subjected to *in vivo* excision. The pBluescript phagemids excised from the λ clones were purified and insert sizes were evaluated by restriction enzyme digestion analysis. One of eleven positive clones, containing a cDNA insert that was approximately 3.4 kb, was selected for further characterization.

DNA sequencing and computer analysis. Double-stranded DNA sequencing was performed by the dideoxy-nucleotide chain termination method[19] using manual (USB, Cleveland, OH) and automated dideoxy sequencing at the Iowa State University DNA Sequencing Facilitity. DNA and deduced amino acids were analyzed using the programs in the GCG software package (University of Wisconsin Genetics Computer Group, Inc., Madison, WI, USA).

Leukocyte isolation and activation. Mononuclear cells (MNC) and neutrophils were separated as described.[20,21] The isolated neutrophils were suspended to 5 X 10^7 cells/ml in Earle's Balanced Salt Solution (EBSS). Cell suspensions were incubated with opsonized zymosan (OZ; 10mg/ml) for 3 hrs and platelet activating factor (PAF; 1μg/ml) for 0.5 hrs, 1.5 hrs, 3 hrs, and 18 hrs along with equal volume of medium. Mononuclear cells were enriched

by buoyant-density gradient centrifugation on percoll gradients (1.084 sp. gr., 400 X G for 40 min), harvested, and washed in phosphate buffered saline (PBS). The washed mononuclear cells were resuspended to 1 X 10^8 cells/ml in RPMI-1640 medium containing 10% fetal bovine serum with antibiotics and antimycotic, and stimulated with pokeweed mitogen (PWM; 10 μg/ml) and concanavalin A (conA; 1 μg/ml) for the same intervals as neutrophils.

Preparation of gene specific cDNA probes and northern blot analysis. Two sets of PCR primers were designed from cDNAs of bovine ICAM-1 and -3 genes. A DNA fragment (307 bp) from bovine ICAM-1 using 5'-TGGAGATAGCTGGGCAGGT (at 1671 bp) and 5'-GTGGAGCACGTTCAGGAC (at 1978 bp) primers was amplified and a 353 bp fragment from bovine ICAM-3 primers, 5'-GCACCGTAATAGGAGCATCCAGT (at 1269 bp) and 5'-GCAGCGAGAGTCACCAAGCCC (at 1613 bp). The amplified fragments were used as hybridization probes for the following experiments. Total RNA from neutrophils and mononuclear cells was extracted using Trizol™ (Gibco BRL, Gaithersburg, MD) and chloroform, precipitated with isopropanol, and washed with 75% ethanol. Twenty micrograms of total RNA from each sample were electrophoresed through a 1% agarose gel containing formaldehyde, blotted to nylon membranes, and probed with ^{32}P-dCTP labeled bovine ICAM-1 and -3 probes separately. Prehybrization and hybridization conditions were the same as described for the cDNA library screening. Membranes were washed twice with 2x SSC/0.1% SDS for 5 min at RT, once with 0.2x SSC/0.1% SDS for 10 min at RT, once with 0.1x SSC/0.1% SDS for 5 min at RT, and finally with 0.1x SSC/0.1% SDS for 5 min at 55°C, then exposed for 24 to 72 hrs to X-ray film for autoradiography.

Results

cDNA encoding bovine ICAM-1. The cDNA sequence of bovine ICAM-1 was 3398 bp with an open reading frame of 1605 nucleotides that encode for 535 aa, starting with a methionine codon at nucleotide 554 and ending with a termination codon at nucleotide 2158

(Fig. 1). The composite cDNA sequence for the coding region of bovine ICAM-1 shows 69% homology with human ICAM-1. The deduced amino acid sequences for bovine ICAM-1 show 57% and 47% identity with human ICAM-1 and bovine ICAM-3, respectively. The bovine ICAM-1 aa sequence contains a putative signal peptide, five Ig-like domains, a hydrophobic transmembrane region, and a cytoplasmic tail, which aligns it with the Ig gene superfamily. It also contains thirteen N-glycosylation sites indicating that the protein is heavily glycosylated. Fourteen cysteine residues in bovine ICAM-1 are found at the same positions as those of human ICAM-1 and bovine ICAM-3.

Ig-like domain comparison among species. The conservation of amino acid sequences is high throughout all five domains of bovine ICAM-1 compared to human ICAM-1 (Table 1). Domain 2 is the most highly conserved domain among species, whereas domain 4 of ICAM-3 sequences (only bovine and human ICAM-3 are known so far) is the most highly conserved domain. When comparing the domains of bovine ICAM-1 and -3, the homology of domain 2 is 94% whereas the others are below 43% (Fig. 2). Bovine ICAM-1 has one serine residue in the cytoplasmic tail, which neither human, canine, rat, nor mouse ICAM-1 or -2 contain any serine residues in their cytoplasmic tails. Furthermore, bovine ICAM-1 contains two Arg-Gly-Asp (RGD) sequences, found mostly in matrix molecules, unlike human ICAM-1.

Expression comparison of neutrophils and mononuclear cells. Study of bovine ICAM-1 expression by northern blot analysis in Fig. 3 and 4 shows that total RNA levels of ICAM-1 in resting MNC are almost undetectable but increase after 3 hrs and 18 hrs upon stimulation with PWM and conA, respectively. Similarly, ICAM-1 total RNA in resting neutrophils was not expressed at all, but was detectable after stimulation with PAF (Fig. 4). On the other hand, bovine ICAM-3 in neutrophils was expressed abundantly in unstimulated and activated neutrophils while ICAM-3 expression was very low in resting MNC and was down-regulated in activated MNC.

Discussion

We have screened a bovine aortic endothelial cDNA library and isolated a cDNA encoding for ICAM-1 which is 3.4 kb in length with a 5' untranslated region of 553 bp, a complete coding sequence of 1605 bp and a 3' untranslated region of 1237 bp. Sequence comparison indicates that bovine ICAM-1 is a member of the Ig gene superfamily containing five Ig-like domains as does ICAM-1 of other species. Comparison to the human ICAM-1 sequence indicates that the overall homology is 69% at the nucleic acid level and 57% at the aa level. The homology between bovine ICAM-1 and ICAM-3 for aa is 47% with striking identity in domain 2 (94%), postulating that ICAM-1 and -3 may be evolved from a common ancestral ICAM gene.

Intercellular adhesion molecule-1 is expressed at low levels on endothelial cells, epithelial cells, dendritic cells, and leukocytes and its expression can be induced and augmented by cytokines (IFN-γ, TNF-α, and IL-1), retinoic acid and lipopolysaccaride[4,22,23] whereas the expression of ICAM-3 is restricted to resting leukocyte and antigen-presenting cells (Langerhans cells).[6,11,24] Elsner et al. separated resting leukocytes into neutrophils and MNC to look at the expression of human ICAM-1 and showed that both neutrophils and MNC constituitively express ICAM-1 mRNA.[25] We could not detect the constituitive expression of bovine ICAM-1 in either neutrophils or MNC. One possibility is that we used total RNA instead of mRNA, which gives less sensitivity. Northern analysis of bovine ICAM-3 expression, however, shows abundant expression in resting and to a lesser extent in activated neutrophils. This contrasts with clear detection of bovine ICAMs in unstimulated MNC. Human ICAM-3 molecules have been reported to be expressed at a high level on resting lymphocytes, monocytes, and neutrophils by flow cytometric analysis.[5]

During transendothelial migration, neutrophils adhere to ICAMs of endothelial cells by means of their β_2-integrins leading to the accumulation of neutrophils at sites of inflammation. It was postulated the ICAM-3 is involved in the initiation of an immune response because of

the observation that adhesion of resting T lymphocytes to LFA-1 on other lymphocytes occurs primarily via ICAM-3 and its expression is much higher than other LFA-1 ligands.[5] Some ICAM-3 molecules were released upon cell activation by enzymatic cleavage similar to L-selectin and the remaining molecules on the neutrophil surface following activation were still capable of sustaining cell adhesion.[26] The functional consequences of ICAM-3 down-regulation on neutrophils are not evident at present. The bovine ICAM-3 might play a role in more recruitment of neutrophils by means of homotypic interactions during acute inflammatory response. Also, it is possible that ICAM-3 might interact with other counter-ligands on the surface of endothelial cells.

Bovine ICAM-1, -3 and human ICAM-3 have serine residues in the cytoplasmic tail, whereas neither human, canine, rat, nor mouse ICAM-1 or -2 contain any serine residues in their cytoplasmic tails. It was reported that serine residues of human ICAM-3 became rapidly and transiently phosphorylated upon cellular stimulation.[27] The serine residue on the cytoplasmic tail of bovine ICAM-1 in MNC might be important after activation, leading to conformational changes as well as signal transduction. Bovine ICAM-3 on activated neutrophils could participate in homotypic aggregation of neutrophils or heterotypic adhesion with other leukocytes resulting from signal tranduction pathways due to the presence of the serine residue in the cytoplasmic tail.

Integrins (mainly β_3, and several β_1) appear to recognize specific amino acid sequence motifs in their ligands. Arg-Gly-Asp (RGD) is found within a number of matrix proteins including fibronectin, fibrinogen, thrombospondin, vitronectin, laminin and type I collagen although not all integrins bind to ligands via RGD-containing domains.[28,29] The RGD sequences of the filamentous hemagglutinin of *Bordetella pertussis* has been suggested to modulate binding of ligands to Mac-1.[30,31] Bovine ICAM-1 contains two RGD sites and murine ICAM-1 has one RGD sequence. Although synthetic RGD peptides fail to inhibit human LFA-1/ICAM-1 mediated adhesion and the synthetic peptide LASRGDHG had no

effect on LFA-1 dependent cell-cell interaction in mice,[16] the importance of these RGD regions in receptor-ligand interaction can not be ruled out.

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Fig. 1. Nucleotide sequence and the deduced amino acid sequence of bovine ICAM-1 cDNA. The hydrophobic putative signal peptide and transmembrane sequences are indicated by a dashed line. The cysteine residues are circled and N-linked glycosylation sites are boxed. The proposed start points for Ig domains are indicated above the nucleotide sequence. The polyadenylation signal AATAAA in the 3'-untranslated region is in bold.

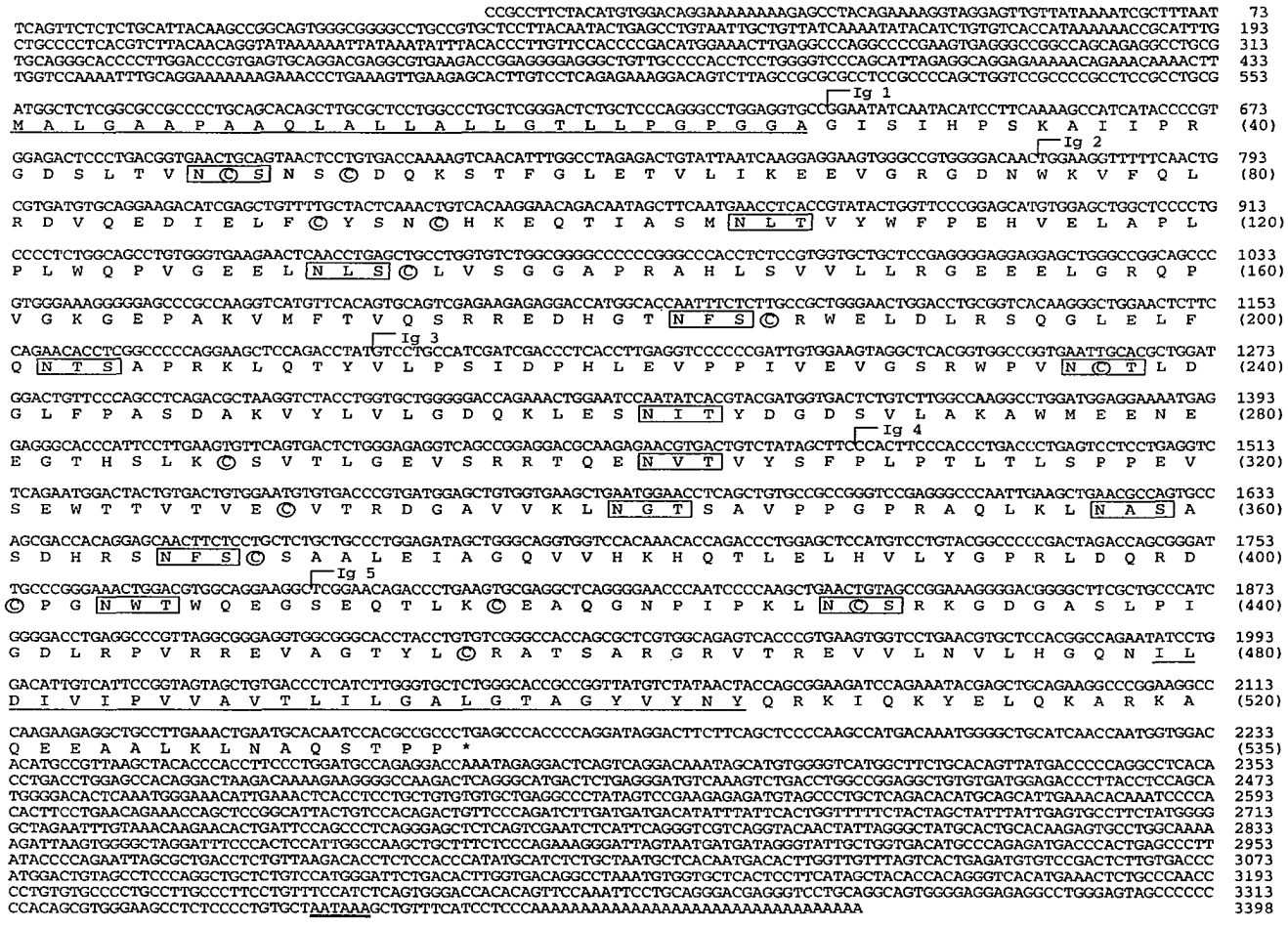
Ig 1
Ig 2
Ig 3
Ig 4
Ig 5

Table 1. Interspecies comparison of amino acid identities among five Ig-like domains of ICAM-1 and ICAM-3.

Comparison	Domain 1	Domain 2	Domain 3	Domain 4	Domain 5
dog/human[a]	55%	**77%**	55%	66%	54%
dog/mouse[b]	49%	**64%**	39%	63%	56%
human/mouse[c]	48%	**64%**	46%	57%	46%
bovine/human[d]	52%	**70%**	48%	62%	51%
bovine/human[e]	57%	64%	67%	**72%**	55%

[a b c.d] Domain identity of ICAM-1 among species

[e] Domain identity of ICAM-3 between bovine and human

Percent of amino acid identity between Ig domains among species was showed. Bold numbers represent highest homology percent within ICAM-1 and -3 domains.

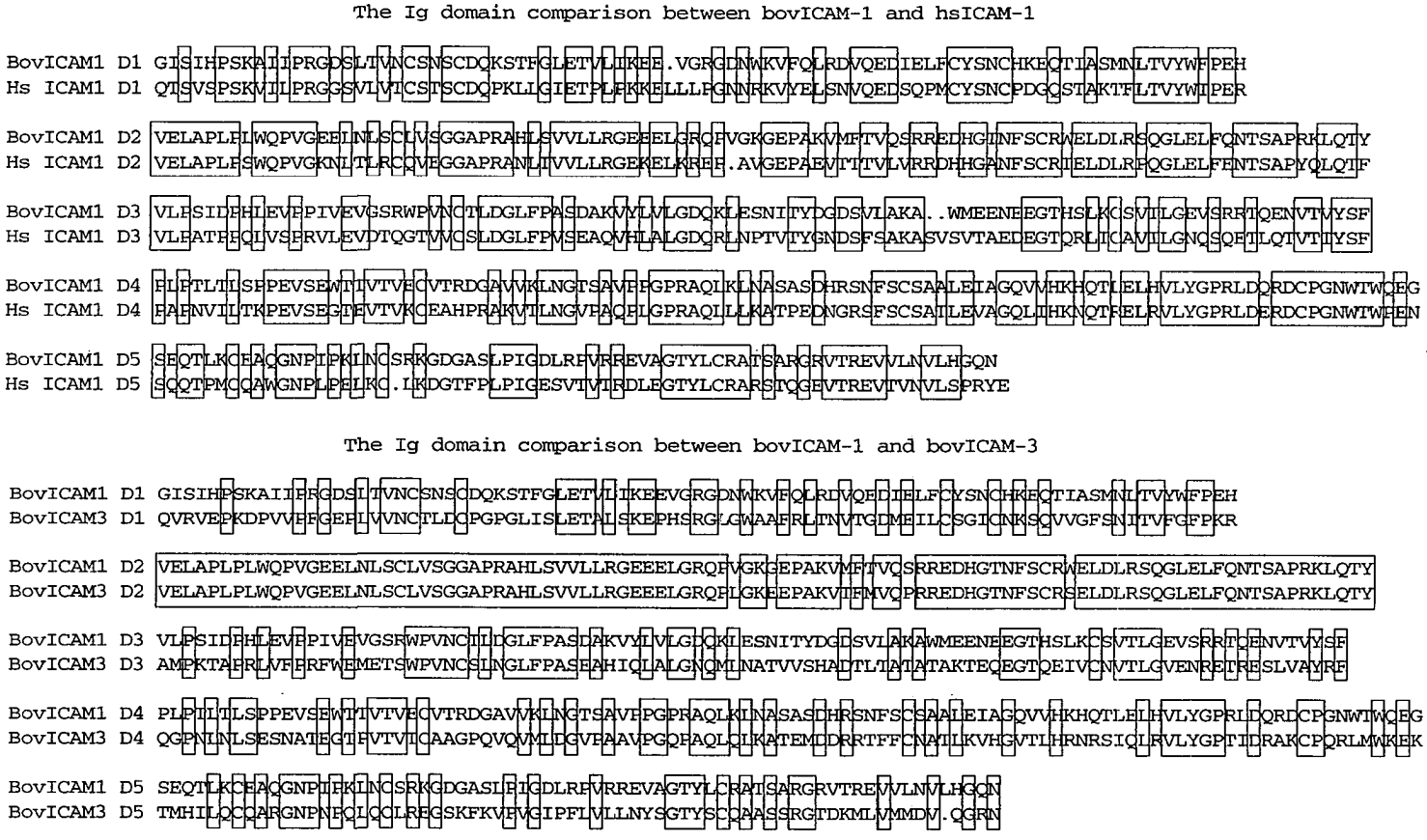

Fig. 2. Homology of bovine ICAM-1, human ICAM-1, and bovine ICAM-3. Alignment of Ig domains between bovine/human ICAM-1 and bovine ICAM-1,.bovine ICAM-3. Alignments of ICAMs were generated using program bestfit from GCG software package (University of Wisconsin Genetics Computer Group).

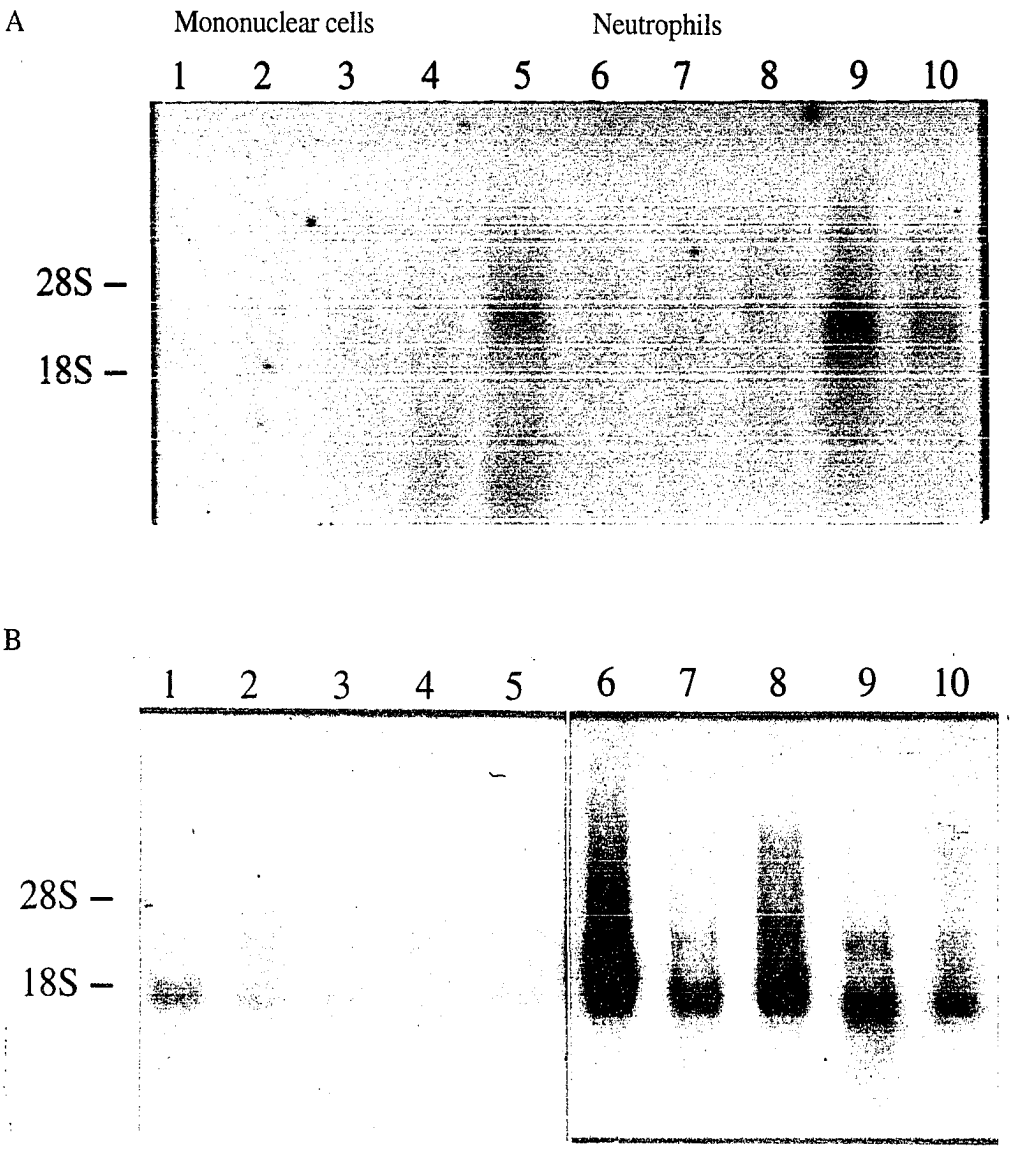

Fig. 3. Northern blot analysis of bovine ICAMs. 20 μg of total RNA from MNC (lane 1-5) and neutrophils (lane 6-10) were electrophoresed and probed with bovine ICAM-1 (A) and -3 (B) fragments. MNC were stimulated with conA (1 μg/ml) and neutrophils with PAF (1 μg/ml) for 0 (lane 1 and 6), 0.5 (lane 2 and 7), 1.5 (lane 3 and 8), 3 (lane 4 and 9), and 18 hrs (lane 5 and 10).

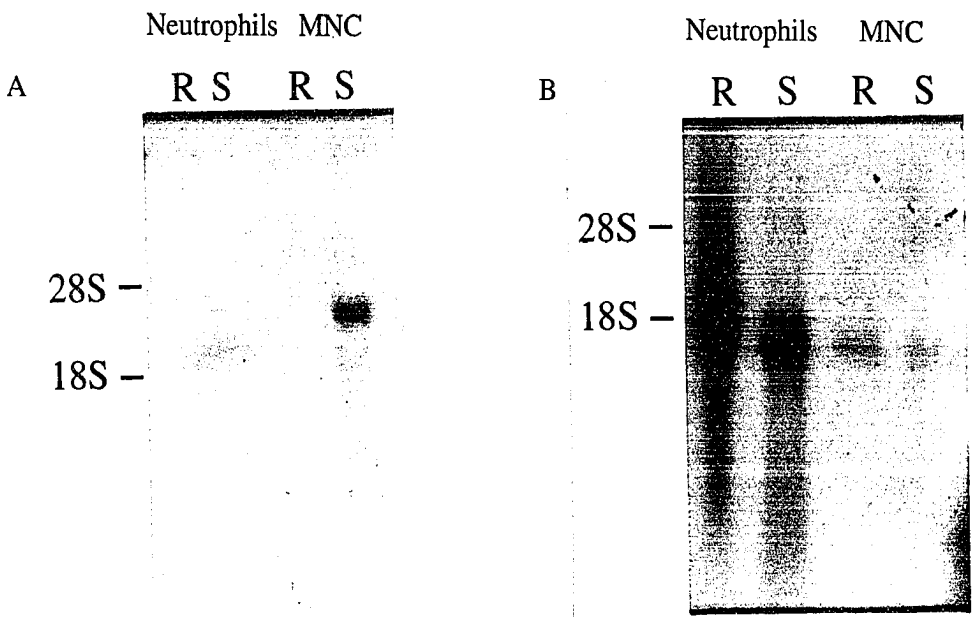

Fig. 4. Northern blot analysis of bovine ICAMs. 20 μg of total RNA used and probed with bovine ICAM-1 (A) and -3 (B). Mononuclear cells (MNC) were unstimulated (R) or stimulated (S) with PWM (10 μg/ml) for 3 hrs. Neutrophils were also unactivated (R) or activated (S) with OZ (10 mg/ml) for 3 hrs.

GENERAL CONCLUSIONS

Leukocytes circulate from blood into tissues as part of their role in the body's defense system against invading microorganisms. Neutrophils must adhere to the endothelial cells and migrate across the vessel wall into the tissues in postcapillary venules at sites of inflammation. The interaction of these circulating cells with endothelium involves multiple families of adhesion molecules and is essential for leukocyte recruitment in diseased or damaged tissues.

Neutrophil recruitment into the inflammation site begins with the local dilation of postcapillary venules, resulting in additional margination of cells as blood flow rate declines. Neutrophils loosely adhere to the endothelium via P- and E-selectin on the endothelium and L-selectin on neutrophils.[1,2] This weak binding results in rolling of the neutrophil along the vessel wall. Binding of selectins to their ligands and chemoattractant release from endothelial cells triggers arresting of neutrophils by enhancing expression and adhesiveness of the β_2-integrin, CD11b/CD18 (Mac-1).[3,4] Mac-1 allows neutrophils to bind tightly to activated endothelium via its ligand, intercellular adhesion molecule-1.[5] Following firm adhesion, the neutrophil migrates across the endothelial wall. They then can continue through the extracellular matrix to the site of inflammation or tissue injury by binding to specific extracellular matrix proteins utilizing β_1-integrins.

The objectives of this study were to determine the expression of adhesion molecules on neutrophils of periparturient cows and neonatal calves and to clone bovine intercellular adhesion molecules. In the first study, adhesion molecules, the β_2-integrins (determined by CD18 expression) and L-selectin (CD62L), on neutrophils of periparturient cows and neonatal calves were characterized by flow cytometry using specific monoclonal antibodies. Periparturient cows are known to be immunosuppressed. Some periparturient and early lactation cows show an increased susceptibility to infections.[6-11] Impaired neutrophil recruitment has been implicated as one of the factors contributing to increased rates of

infection. Also, immature host defense mechanisms have been cited as one of the most important causes of increased susceptibility of neonates to infection. The ability of neutrophils to adhere and migrate, which is dependent on the integrin adhesion molecule family has been reported to be abnormal.[12-14] If neutrophils from periparturient cows and neonatal calves have an impaired ability to migrate to sites of infection, the initial adhesion molecule interactions might contribute to the impairment. Therefore, we questioned whether the adhesion molecules on neutrophils are changed in periparturient cows and calves. We observed low expression of CD62L on neutrophils from cows as well as CD62L and CD18 on calf neutrophils for several days postpartum. This down-regulated expression may result in impaired inflammation, thus contributing to the susceptibility to disease in dairy cows and calves at this time.

The numerous reports of high concentrations of plasma cortisol in periparturient cows and one day old calves have been associated with the increased incidence of infectious disease. Although we did not measure cortisol concentrations in blood, the decreased expression of CD18 and CD62L molecules on blood neutrophils after parturition could result from elevated cortisol concentrations since in vivo glucocorticoid administration to Holstein cows induces down-regulation of CD62L and CD18 expression on blood neutrophils.[15] However, the high expression of CD18 at parturition, coincident with peak endogenous glucocorticoid levels is paradoxical. It is not likely that reduced adhesion molecule expression is the result of a change in a single factor. During pregnancy, many steroid and peptide hormones change; some hormones could cause increased CD18 expression on neutrophils. This might be explained by complicated relationships among the endocrine and immune systems and their net effects on adhesion molecules. It might be difficult to distinguish which factor is contributing to the resultant expression of adhesion molecules. On the other hand, expression of CD62L is entirely consistent with the changes being mediated by increased endogenous glucocorticoids. Dramatically decreased expression of CD62L on neutrophils at parturition may cause diminished egress of neutrophils from blood into tissues of cows and neonatal calves.

Although expression of adhesion molecules is essential for neutrophil adhesion and migration, simple expression of these molecules does not determine the functional capacity of cells to adhere and migrate in vivo. Since it is known that hyperadherence by neutrophils is dependent not only on the quantity of CD11b/CD18 translocated to the cell surface[16] but also on altered conformational changes of CD11b/CD18 for its ligand under continuously increasing chemotactic stimulation,[17] further research is needed to determine the functional capacity of neutrophils (adherence and transendothelial migration assays) of periparturient cow and calves utilizing bovine endothelial cell cultures and chemotactic factors.

In the second and third experiments, we cloned, sequenced and analyzed two intercellular adhesion molecules (ICAM). A partial bovine ICAM-3 gene was obtained by screening a bovine mammary gland cDNA library. A full-length clone was then obtained by polymerase chain reaction amplication of a bovine lymph node cDNA library. The cDNA of bovine ICAM-3 contains 1827 bp encoding 544 amino acids. The deduced amino acid sequence from the coding region shows 61% identity with human ICAM-3. It contains twelve cysteine residues at the same positions as those of human ICAM-3 and fifteen N-glycosylation sites. Then, using a DNA segment from the bovine ICAM-3 cDNA, we isolated a bovine ICAM-1 gene from a bovine aortic endothelial cDNA library which shall not express ICAM-3. The 3398 bp of the bovine ICAM-1 cDNA sequence codes for 535 aa and shows 58% homology at the amino acid level to human ICAM-1. The predicted number and positions of cysteine residues in bovine ICAM-1 are all conserved among species and with bovine ICAM-3. Both bovine ICAM-1 and ICAM-3 amino acid sequences contain a putative signal peptide, a hydrophobic transmembrane region, and a cytoplasmic tail. They are also predicted to be heavily glycosylated and consist of five immunoglobulin-like domains, which aligns them as members of the immunoglobulin gene superfamily. Northern blot analysis results show that the bovine ICAM-1 gene is expressed in stimulated leukocytes and bovine ICAM-3 predominantly in resting neutrophils.

Inhibiting extravasation is a useful tool of limiting neutrophil-mediated tissue damage. The multiple steps of adhesion during neutrophil migration can be blocked by monoclonal antibodies and soluble proteins. Monoclonal antibodies have been used in animals and humans to prevent excessive infiltration of tissue by inflammatory cells by blocking the function of particular adhesion molecules.[18] For example, antibodies to ICAM-1 reduced tissue infiltration and damage by neutrophils.[19] Obviously, the use of monoclonal antibodies in the treatment of human disease is limited by the generation of antimurine-immunoglobulin antibodies by the recipient. Because of this problem and the potential for causing immune complex disease, soluble forms of adhesion molecules are also being considered for blocking cellular adhesion. Soluble forms of human ICAM-1 have been produced in prokaryotic and eukaryotic systems and have been shown to block leukocyte adhesion.[20-22] Now that we have cloned two ICAMs, future studies to produce various forms of soluble bovine ICAM and studies to demonstrate their roles in leukocyte adhesion are possible. Once their roles in adhesion are known then the utility of soluble bovine ICAMs as the next generation of anti-inflammatory drugs can be explored.

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